

nature

1 June 1978

UNCSTD: a survival exercise for the developed world?

THE countdown to the United Nations Conference on Science and Technology for Development (UNCSTD) in August 1979 calls for national papers to be submitted to the secretariat by 1 May this year in first draft, by 1 August in final draft. Needless to say this first deadline has been missed by many nations, but there is a widespread flurry of activity at present, as papers begin to emerge or at least to be circulated internally for discussion. Some developed nations have entered into this exercise with enthusiasm—the Swedes and the Canadians are likely to bring to UNCSTD the same vigour they bring to many international meetings, particularly as both nations, free of ex-colonial attachments, have developed special institutions aimed at experimenting in development assistance (Wendy Barnaby reports on Swedish preparations on page 328). But for most of the developed world UNCSTD is no more than something that has to be lived with and survived intact, so national papers are likely to be relatively bland and non-committal. This will contrast with papers from many developing countries in which radical and unambiguous viewpoints are going to be expressed.

The British national paper will, it is widely expected, be in the cautious mould adopted by most developed countries. It is likely to describe quite carefully the way Britain runs its science, the way British assistance for developing countries is channelled, the sort of general priorities that are assigned to different aspects of aid. It will, no doubt, point out that the aid programme for the next three years is scheduled to grow by 6% per year from its present level of around £700 million net annually. The sentiments expressed on the need for adequate technical manpower within the developing world will doubtless be totally unexceptionable.

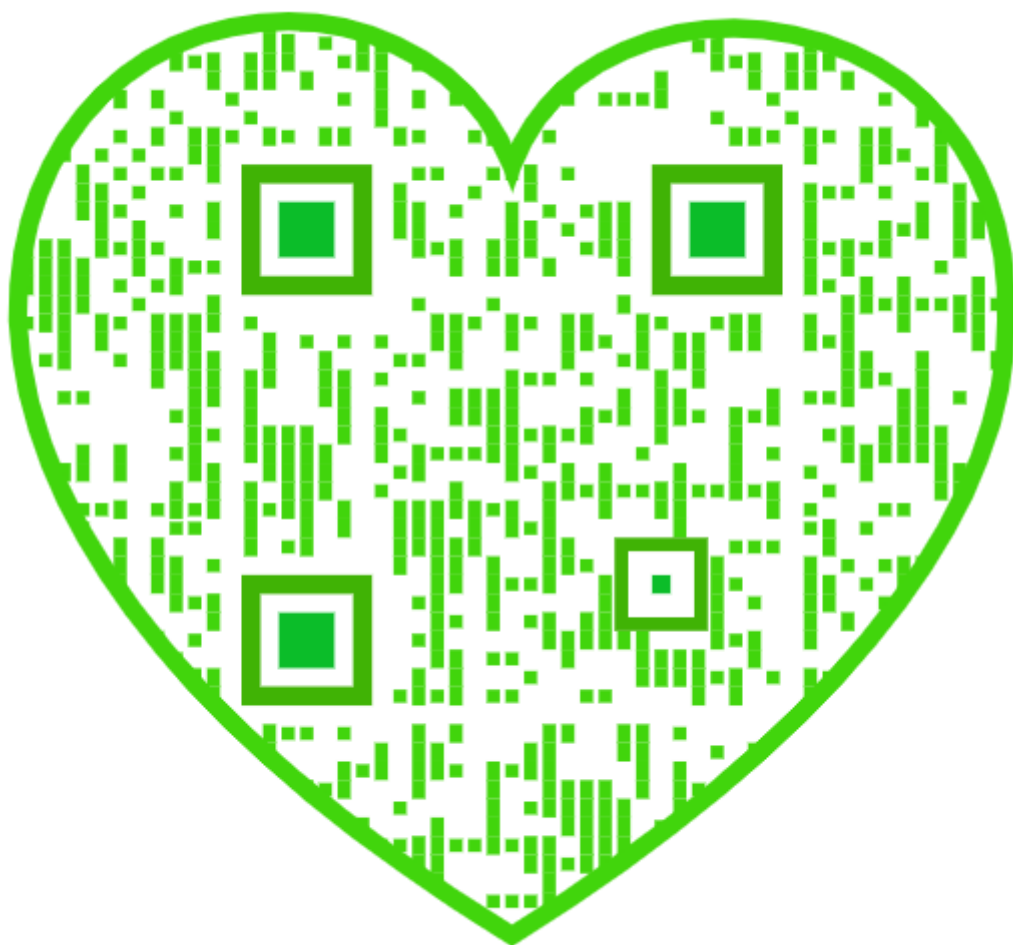
One issue which will be carefully avoided, however, is that of industrial development. There can be general agreement that science and technology should contribute to health, agriculture and exploitation of natural resources, and few in the developed world would stand in the way of that sort of aid. But what about encouraging local industry to expand and provide some of the products which traditionally Britain has supplied? Here we run into deeper political waters because one country's development may mean another's unemployment. So is the developing world simply to remain the supplier of raw materials? It would have been good if the Ministry of Overseas Development had been able to grasp that nettle more firmly. But instead there will be an air of 'damage-limitation' about the whole report. From the British government's point of view there

is, alas, nothing to be gained and a lot to be lost by floating new ideas in a document like this. At UNCSTD anything that is regarded as preaching will be met with hostility; anything that suggests new commitments will be met with calls for immediate implementation.

Not so shackled are the non-governmental organisations—indeed several forthcoming conferences by scientists themselves on development are likely to be quite lively affairs. And numerous national academies of science are expected to contribute discussion papers to the UNCSTD secretariat alongside the official national papers. The Royal Society is one such, and although its paper has been put together in an indecent haste (it is less than two months since its working party first met) there is a good chance that the report will venture into territory which the national paper could only touch on rather lightly—the quality of technical staff.

There were some raised eyebrows when the working party—average age 66—was announced. Wouldn't it all look a little too senior? But this very seniority has allowed the group to report, from lifetimes of experience, on the difficulties of transplanting ideas and technology when the ground into which they are being transferred lacks middle levels of expertise who will actually have to cope on a day-to-day basis. Preaching? Maybe, but the chairman, Sir Ieuan Maddock, would say much the same thing about trends in the developed world—that there are a diminishing number of people prepared to make, do, operate and repair, and this bodes ill for manufacturing industry. The remedy cannot be universal; it must be specific and depend on whether developing countries want to go it alone or take advantage of bi- or multilateral aid. But it is bound to need higher standards of technical training, a loosening of the idea that a university degree is the only qualification worth having (and immediately excuses the holder from any dirty work) and the strengthening of ancillary services such as libraries and information departments.

The Royal Society's contemplative paper is likely to be in contrast to that from the US National Academy of Sciences. At last count, the academy was proposing 22 areas of initiative in food, health, resources, urban and industrial problems, in which the theme of expansion recurred with monotonous regularity. With the best will in the world one is left wondering whether the expansion would go beyond that of job opportunities in the US. The proposals sound too much like plans for a crusade against underdevelopment, and may well attract little support outside the United States. □



Sweden's view of development science

Sweden devotes a larger fraction of its gross national product to foreign aid than almost any other member of the United Nations, so it has taken its contribution to the forthcoming United Nations Conference on Science and Technology for Development very seriously. **Wendy Barnaby** reports

SWEDEN is finishing preparing its national report on the role of science and technology in development which, with the other 148 United Nations members' national reports, will be circulated in preparation for next year's United Nations Conference on Science and Technology for Development (UNCSTD). Inundated as they will be, few delegates will bother to read any given report. This is a pity, for Sweden is bending over backwards not to preach. Its report is a plea for a particular outlook on science and society. And its starting point is what it has learned about the role of science and technology in its own development.

The main concern is that UNCSTD should not degenerate into a discussion of the difficulties of various technological fixes that have been exported to Third-World countries. Sweden would like to see the conference deal more widely with development problems, and the ways science and technology interact with them. It hopes for more awareness of the way science and technology affect the whole process of development. So, drawing on its own experience, it calls attention to what has to happen in any society before research and development can find a footing there: political decisions must be made to start R & D; specific sums must be allocated to specific projects by officials in an organised way; trained scientific personnel must coordinate their labour to carry out the projects; and the scientists must have been produced by an educational system anchored in the local culture.

Sweden sees science and technology as one link in this chain (cultural values—educational system—scientific-technological infrastructure—bureaucrats—politicians), and stresses that all the links affect each other. So if any country is suddenly injected with a certain sort of technology, for instance, there will be consequences for all the other aspects of that society, not merely for its technological mix.

According to Mr Lennart Baage, of the Foreign Ministry's Office for International Development Cooperation, the national report will make some general proposals in keeping with this outlook. A summary of the report makes various suggestions for establishing efficient R&D in any society: that bodies in the central state administration should be created to stimulate and plan for R&D; that results should be

given a means of application through an infrastructure, built up from an education system; that R&D resources should be devoted to priority areas relevant to social goals; that the development of a domestic capacity for technological evaluation may be an important step towards self-reliance; that the pace of structural transformation historically associated with development in industrialised countries



should, if possible, be avoided in the Third World; and that goals for major R&D projects should be clearly specified so that the success of the projects can be judged.

The summary says that development should be supported first in the poorest countries, on an international level, and urges better coordination of science and technology within the UN system and a clearer division of responsibilities between the agencies involved. Baage says that no proposals for specific countries will be made before Sweden has heard what various Third-World countries themselves have to say.

UNCSTD is being treated with customary Swedish solemnity. A lot of (also customary) hard work is going into the preparations. The national report was arrived at by subjecting various commissioned studies for review by a panel of representatives from several interested bodies. It carefully defines the concepts of 'science', 'technology' and 'development' before elaborating on the Swedish outlook. Its pedantry, logic and pleas for a holistic approach must seem a luxury to the Third World. Yet the Swedish

view has resulted not only from domestic experience, but also from attempts to get to grips with some of the problems facing scientists in developing countries.

The largest umbrella for these attempts is the Swedish Agency for Research Cooperation with Developing Countries—SAREC. One of the institutes it supports is the International Foundation for Science (IFS), also based here. This small group gives grants to young scientists from developing countries on the conditions that their work is relevant to the needs of their country and carried out in it or some other part of the Third World. This year, IFS is dividing about \$1.3 million between 250 projects. Sweden supplies half of its budget, and the rest comes from state grants from eight industrialised countries and one developing one—Nigeria. More research with an eye to the Third World is being done through the international seminars in physics and chemistry at the University of Uppsala. Each year, the seminars give grants to 30 young thirdworld scientists who must have been employed by a university in their home country for at least one year: this stipulation being an effort to stop the brain drain. The Fellows join a Swedish research group in their field of interest. About 90% of the Fellows are still active in their home countries several years after having been maintained by the contacts and visits that the Swedish researchers are generally able to keep up with them.

According to one study done for the national report, about a third of Sweden's approximately 900 research institutes are doing research which they judge to be of direct interest to developing countries, although only about 10% actually cooperate with the Third World. About 400 projects were cited as being directly relevant to developing countries: 42% are in the social sciences and humanities (mostly related to Asia); 22% in medicine (mostly related to Africa); 17% in mathematics and natural sciences (again mainly related to Africa) and 10% in agriculture, forestry and veterinary science (with Africa slightly more popular than Asia).

There is a lot of research being done in Sweden which could possibly be useful to the Third World. This may not be surprising given recent estimates that 62% of world R&D resources are spent within OECD countries, 34% within Comecon and only 4% within developing countries, where even this minuscule amount is concentrated in only a few. But how are developing countries assured access to research that could help them, given

that it is practically always done in industrialised countries? This is one of the subjects to be taken up at a conference in May and June organised by the research policy programme at the University of Lund. Created in the late 1960s to study questions of science and technology policy, the research policy programme has focused attention on UNCSTD and, through the international distribution of its SAREC-supported 'Lund letter', which chews over the problems that the conference will hopefully tackle, has stimulated discussion on the issues involved. Researchers at Lund agree with people at SAREC and those working on the national report: all stress the importance of preparatory work and discussion, which they hope will prevent UNCSTD from becoming a huge jamboree with no coherent argumentation or follow-up.

The question of appropriate technology is also widely discussed. The national report goes out of its way to point out that, in a reaction to the belief that complicated technology will solve the Third World's problems, many people regard appropriate technology as essentially soft or simple. But, it stresses, technology that is suitable for a particular job in a particular country may be either simple or highly complex, depending on the capacity of the receiving country to use it and adapt it to its social and economic conditions. The question is complicated, because the degree of adaptation needed is not obvious and may be influenced by all sorts of factors. A survey of 15 large Swedish companies with international branches has shown that they have made only a few attempts to adapt their technology to suit the developing countries they operate in. This was said to be partly because some of the technology needed no modification, and partly because of the status-consciousness of some of the recipient countries.

Although the national report does not mention the fact, Sweden is one of the few UN members which contributes 1% of its gross national product in foreign aid. As a recent government statement points out, however, aid is only one part of trying to fulfil development goals. There is not much hope of alleviating the worst problems in many Third World countries until the price of raw materials has been stabilised, debts written off, and entrance assured to goods and capital markets. The Swedes are convinced that a similarly broad picture of the role of science and technology in development must be kept in focus if UNCSTD is going to make any difference at all to the two-thirds of the world's population in the developing countries. □

News from Orlov's trial

The Moscow Helsinki Monitoring Group put out this statement last week. It is based on Irina Orlova's account

“ORLOV'S trial was a fiction. This is obvious from the fact that the authorities needed to detain him for 15 months in conditions of the strictest isolation in order to prepare the trial and only three days to examine the materials of the investigation in 58 volumes. The court needed no more than a few hours to discuss and draw up the sentence. . . .

The formally open trial took place behind closed doors. Apart from specially selected people only Orlov's wife and two sons were allowed into the court room. Each time they were subjected to humiliating searches. Orlov's wife was stripped naked in the presence of men. Orlov's son was several times beaten on the head. Orlov's friends, including Academician Sakharov, were not allowed into the court room. . . .

In the course of the trial the Procurator and the court carefully avoided mention of the fact that the documents incriminating Yu Orlov are documents of the Moscow Helsinki Monitoring Group. The court established only the fact of Orlov's participation in the compilation of the documents and suppressed all attempts by Orlov and his counsel to examine the documents with regard to their content. That is, the court regarded all the documents in advance as “slandorous”. . . .

All appeals by Orlov and his counsel directed towards defending Orlov—the calling of witnesses for the defence, making known documents—were rejected by the court. Orlov's evidence was persistently and repeatedly interrupted by the court and his counsel's questions were struck out by the judge.

Realising that the advocate Ye S. Shalman could not carry out a political defence, Orlov, at the end of the judicial investigation, expressed gratitude to the counsel for his legal and moral help and renounced participation by the counsel in the cross examination, declaring that he himself would make the speech for the defence.

However, Orlov's speech for the defence was interrupted by numerous shouts from the judge and hostile cries

from the specially selected audience.

The judge also interrupted the final speech. Orlov said: “Are you not ashamed to interrupt me, after all this is my last word?” However, even after this he was denied the chance to speak without interruption. Orlov was not allowed to complete either his speech for the defence or his final words.

After the court had granted the request that the counsel be relieved of any further participation in the case, the counsel, Ye S. Shalman, was removed by brute force from the court and was permitted to return only after he had telephoned the directors of the college of lawyers. . . .

Therefore we affirm that the trial of Orlov was not an objective and just investigation but an attack on freedom of thought and speech.

The significance of the trial of Yuri Orlov, as well as that of political trials which have taken place in the past and those which are expected to take place in the near future of his friends in the Moscow Helsinki Group, the writer A. Ginsburg and the cybernetician A. Shcharansky, extends far beyond the borders of the USSR.

These trials have a direct relation not only with the question of human rights but also with that of detente in international relations.

We call upon the governments and heads of state of countries which signed the Helsinki Act, public organisations of those countries and private individuals, in the first place scientists and writers, to speak out in defence of Yuri Orlov, in defence of the Helsinki Act itself, which asserts an indissoluble link between the problem of security and human rights.

Members of the “Helsinki” Group: Y. Bonner, S. Kalistratova, M. Land, N. Meyman, V. Nekipelov, T. Osipova, V. Slepak. Member of the Georgian “Helsinki” Group: M. Goldshteyn.

We fully support the statement of the “Helsinki” Group about the trial of Professor Yu F. Orlov: A. Sakharov, I. Nudel, S. Polikanov, A. Lavut, A. Polikanov, I. Kovalev, Yu Yarym-Agayev, L. Kopelev, V. Kornilov. ”

The EEC reaction to the trial

“The Nine, who consider that the Helsinki Final Act constitutes a programme of action for detente recall that, in this document signed by their heads of state or government, the participating states have committed themselves to respect human rights and fundamental freedoms and have confirmed the right of the individual to know and act upon his rights and duties in this field. . . . The governments of the Nine find it incompatible with the Final Act and with detente that individuals should be prosecuted and sentenced for having demanded the implementation of the Final Act in their own country”.

US refuses nuclear fuels to South Africa and Pakistan

THE US state department has withheld approval of licences for the export of highly-enriched uranium to South Africa and of plutonium to Pakistan, because of concerns about the intentions of each country with regard to the development of nuclear weapons.

The South African request was for 57 pounds of highly-enriched uranium for its Safari research reactor, and was initially filed three years ago. According to the State Department, approval of the licence has been held up "awaiting the outcome of a review of US-South African nuclear cooperative relationships."

In the case of Pakistan, the request was for less than a pound of plutonium which was destined for a research reactor for use in spectrometry research. The State Department has expressed its concern that Pakistan has announced its intention to obtain a reprocessing facility for spent nuclear fuels, which could make it possible for the country to produce enough plutonium for use in nuclear weapons.

Under the terms of the nuclear non-proliferation act, which was signed by the President in March, such factors

must now be taken into account in deciding whether or not to grant an export licence for nuclear fuels.

Meanwhile the US is still maintaining its embargo on the export of nuclear fuels to the member countries of Euratom that came into effect last month following Euratom's refusal to renegotiate its existing agreement on nuclear fuel exports.

Sources in Washington said that pending further moves from Brussels, the existing stalemate—which results largely from complaints that the US has acted unilaterally in revoking the existing agreement—could last through to the autumn.

In Brussels the opposition to renegotiation comes principally from France. The other 8 signatories of the Euratom treaty—Britain, Germany, Italy, Belgium, Denmark, Eire, Luxembourg and the Netherlands—are all agreed that a letter could be sent to President Carter expressing willingness to renegotiate the agreement.

France is expected ultimately to capitulate to pressure from the other members to send such a letter. Meanwhile, the problems caused to Europe by the US embargo do not appear to

be acute, but they are growing. Half of Europe's medium-enriched uranium comes from the USSR. Nearly all the highly enriched uranium, used in research reactors, comes from the US but "It's not like stopping milk or steel" said a Brussels spokesman last week. "The nuclear supplies are needed only infrequently".

Last year Europe withstood a 9-months' stop on the US supply of enriched uranium, while President Carter gathered his thoughts on the issue. But there were special factors operating then. One of the most affected reactors was the high flux neutron beam reactor at the Institute of Laue Langevin at Grenoble. The head of administration at ILL, Dr W Grillo, said last week that the laboratory could not have continued operating—had it not been for a special allowance of highly enriched uranium supplied by the US because ILL was an international organisation.

Dr Grillo believes that if the present embargo continues, ILL will need to look for a similar concession before long. "The situation is not good," said Dr Grillo. "The American regulations are leading to big delays". □

US government to review industrial innovation

CONCERNED at the apparent relationship between declining industrial productivity and growing inflation, the White House announced last week that it has initiated a major review of the effect of government policies on technological innovation. The review will cover the activities of all the major government departments and agencies, and is expected to lead to a number of policy options which will be placed before President Carter sometime next year.

The review is largely the outcome of recent studies carried out by Dr Frank Press's Office of Science and Technology Policy, which has been focusing much of its attention on the relationship between economic performance and national investment in research and development. It was on Dr Press's initiative, for example, that the Office of Management and Budget agreed on the need for a significant 11% increase in support for federally sponsored R & D which the President requested in his budget proposals for the fiscal year 1979 presented to Congress in January.

A number of issues have given rise to the administration's concern at the current health of industrial innovation in the US.

These include:

- indications that industry tends to 'underinvest' in innovation;
- an increased emphasis in private R & D in recent years on low-risk, short-term projects directed at incremental product changes rather than at longer-term research leading to new products and processes;
- the declining international competitiveness of some segments of US industry;
- difficulties that small, high-technology firms encounter in obtaining venture capital.

The situation is illustrated by figures recently released by the National Science Foundation which show that since 1970, industrial R & D expenditure in constant dollars has increased by less than 1% while industrial expenditure on basic research has fallen by 1%. The NSF also comments that "barring continued federal increase, basic research in industry is expected to remain constant, in real terms, and could possibly decline".

The innovation study will not be carried out by OSTP, but by an industrial innovation coordinating committee under the chairmanship of the Department of Commerce. It will concentrate on all aspects of federal policy that affect industrial R & D and innovation, focusing in particular on any "negative

impacts" of such policies which might be reduced in a way consistent with other national goals.

This is a direct reference to growing complaints from industry that proliferating government regulations in fields such as environmental and safety controls are one factor that has been inhibiting R & D efforts, much of which has consequently become "defensive" rather than offensive.

However, speaking at the Massachusetts Institute of Technology last week, Dr Frank Press said that since World War II there had been a dominant pattern by which the federal government supported basic research in universities and federal laboratories leading to the applied R & D which were phased into commercialisation by the private sector. Although this system had worked well, Dr Press said that it was now "essential that we re-evaluate it and see how it might be improved".

Although the precise form of the study has not yet been determined, it is expected that in addition to the studies that will be carried out by individual departments and agencies, arrangements will be made at a relatively early stage for public discussion of the issues involved.

David Dickson

US safety inspectors barred from making spot checks

LAST week was a busy one for the Department of Labour's Occupational Safety and Health Administration. On Tuesday, the Supreme Court, in a 5-3 ruling, decided that OSHA inspectors must obtain a warrant before inspecting premises suspected of breaking safety and health regulations. On the same day, it was learnt that President Carter's economic advisers had ordered the agency to delay the publication of regulations covering occupational exposure to cotton dust in the cotton industry on the grounds that the regulations might have an inflationary impact on the economy.

And the General Accounting Office, the investigative branch of the US Congress, published a report claiming that many serious safety hazards are overlooked by OSHA inspectors, and that 25 states which operate their own OSHA-approved health and safety enforcement programmes are doing an inadequate job.

The Supreme Court decision was the result of a case first brought by OSHA in 1975 against a heating and plumbing contractor in Idaho who had barred an OSHA inspector from entering his premises without a warrant, claiming it to be an unconstitutional invasion of privacy. The Supreme Court, to which OSHA had appealed an earlier court ruling, agreed that this was so and that OSHA inspectors required a warrant to carry out inspections. However the court added that it was not necessary for OSHA to meet strict criminal law requirements in order to obtain such a warrant.

Dr Eula Bingham, the head of OSHA, admitted later that the agency might have to alter its inspection procedures, but said that the decision was sufficiently flexible to permit the routine issuance of warrants if employers refused to allow voluntary inspections.

Mr A. L. Grospiron, however, president of the Oil, Chemical and Atomic Workers and chairman of the AFL-CIO's standing committee on safety and occupational health, said that the decision would give companies time to shut down unsafe operations before inspectors arrived, and that it would "place another roadblock in the way of effective enforcement in the occupational safety and health laws".

The delay in implementing regulations covering exposure to cotton dust, which would affect over 800,000 workers in the cotton industry, follows a report from the council on wage and price stability that OSHA's proposals



"It's good to know that our sick lungs are helping to fight inflation"

would cost industry \$625 millions in initial capital expenditure and a further \$200 million a year.

Although OSHA had previously said that the regulations would be published by 31 May, Charles L. Schultze, chairman of the president's Council of Economic Advisers, has ordered a delay, saying that he wants the proposal to be reviewed for its likely economic impact.

David Dickson

Genetic engineering guidelines: Europe hopeful about collaboration

INTERNATIONAL accord, if not agreement, emerged from a meeting of the European Science Foundation's genetic engineering liaison committee last week. Despite the presence of a representative of the US National Institutes of Health, which have been expected to relax their containment controls on recombinant DNA research in the next few months, delegates left the meeting with the feeling that no one country was likely to take drastic unilateral action in the near future. Whether the NIH are misleading their European colleagues, or whether the relaxation they are contemplating will in the event be only a minor one, is unclear.

One delegate thought that there had been a "considerable convergence of opinion" at the meeting on how far countries could go towards relaxation, which "would have to be ushered in quite slowly". More scientific evidence and experiments relating to safety would have to be accumulated before

there could be any substantial changes in regulations.

Meanwhile in the UK the Health and Safety Executive hope to have their order for compulsory notification of recombinant DNA experiments laid before parliament early this month, and on 15 June the genetic engineering sub-committee of the Select Committee of Science and Technology will meet for its first private briefing with scientists. It will begin to take evidence in early summer, most likely beginning with the Genetic Manipulation Advisory Group. The Department of Education and Science might follow. Then it seems quite possible that a general election will intervene, when the committee would have to be re-constituted.

Robert Walgate

Scientific cooperation under attack

SCIENTIFIC cooperation agreements have over the last few years become almost a ritual concomitant of any discussions of détente. Last week, however, the Seventh British-Soviet Joint Commission found that public endorsement of such agreements can no longer be entirely divorced from the wider issues of the Helsinki accords.

The London meeting commenced only a few days after the sentencing of physicist Yurii Orlov for allegedly slandering the Soviet State. Outside the closing press conference—the sole chance for representatives of the media to meet the Soviet delegation—a determined posse of protesters demanded "No scientific links with Russia". Inside, Academician Vladimir A. Kirillin, Deputy Chairman of the Council, of Ministers of the USSR, and the Rt Hon Edmund Bell, Secretary of State for Trade, exchanged formal compliments and thanks.

Once, however, the meeting was thrown open to the floor, the atmosphere changed. The very first questioner made it quite clear that the Orlov issue outranked any interest in the promising scientific and technological progress noted in the hand-out. "I'm not a man to be scared" claimed Kirillin "so I'm going to give you a rather brief reply". Forty minutes later, under a barrage of incessant questions, he was still replying.

Stripped of its verbiage, Kirillin's answer was simple: the British public is not properly informed about what is going on; otherwise they would realise that Orlov was tried not for his convictions but for breaking the law. In Mr Dell's opinion, however, the British public has a "rather satisfactory" knowledge of Soviet policy on these matters.

Vera Rich



Within a week of Parliament giving the go-ahead for the world's largest nuclear fuel reprocessing plant at Windscale, Cumbria, Britain and Japan signed a £500 million contract which will help pay for the plant and give Japan the right to transport 1600 tonnes of spent fuel to the UK. The plant is not yet built, however, and opposition to it among an increasing number of MPs has been both strong and vocal. Here **Nigel Forman**, MP, Joint Secretary of the Conservative Back Bench Energy Committee, voices his own disquiet



The Windscale error

ON Monday 15 May 1978 at 10 pm the House of Commons decided by a vote of 224 to 80 to give the go-ahead necessary for the development of the planned Thermal Oxide Reprocessing Plant (THORP) at Windscale. The issues involved were both complicated and controversial, but the decision itself was clear-cut and of lasting significance. It set the seal on many months of confused and sometimes angry debate, but it also signified a new degree of nuclear commitment for this country.

At the beginning of his Report on the Windscale Inquiry, Mr Justice Parker posed three key questions:

- Should oxide fuel from UK reactors be reprocessed in this country at all;
- if so, should such reprocessing be carried on at Windscale;
- if so, should the plant be about double the estimated size required to handle UK oxide fuels, so that the spare capacity could be used for reprocessing foreign spent fuel?

These were not really the right questions to ask. The key questions should have been:

- should British Nuclear Fuels Ltd (BNFL) be allowed to build an 'economic' THORP at Windscale;
- if this was not feasible, should it be allowed to build an "uneconomic" plant;
- are there in any case such strong objections to reprocessing on any basis that it should be delayed or even foregone?

The idea of building an 'economic' THORP at Windscale is based upon BNFL's expectation that there will be about £600m worth of foreign business which it will be able to exploit on a cost plus 25% basis in the early 1990's and beyond. It is also dependent upon the assumption that BNFL will get a continuous flow of foreign orders on a comparable basis beyond those which are already in prospect. The economic attractions of THORP can be summarised as getting the Japanese and other foreign customers to sub-

sidise the capital costs of the plant, securing some additional foreign currency over a period when we should not need it quite so much as in recent years, and spreading the costs of our own reprocessing programme over a larger through-put (up to a planned maximum capacity of 1200 tonnes per annum) so as to secure lower unit costs and hence less of a burden from this source on electricity prices.

If these assumptions prove to be reliable—and we should bear in mind that it was BNFL which argued that they will be—the main question under this heading should have been whether or not the assumed economic benefits of THORP are likely to prove sufficiently reliable and attractive to outweigh the non-economic costs and disadvantages. In Para 9.17 of his Report, Mr Justice Parker took the view that "it is as yet too early to reach any final conclusions on the economic position". The proper verdict, supported by the findings of the recent MITRE Report, ought, therefore, to have been the Scottish judicial formula 'not proven'.

However, there is also an argument for building an 'uneconomic' plant. It is based upon the contentions that we already have to reprocess the spent fuel from our own Magnox and AGR stations, that reprocessing is the best available technique of waste management, and that doing it under high standard controlled conditions at Windscale will reduce rather than increase the risks of further nuclear proliferation in the world.

Powerful though it appears to be, such an argument is based upon a number of questionable assumptions. First, there is the assumption that BNFL needs a new plant with the capacity of THORP to deal with the spent fuel arising from our existing nuclear programme. Second, there is the assumption that it is necessarily preferable to reprocess rather than store the spent fuel. Third, there is the assumption that BNFL is wise to reject even more radical alternatives, such as the Linear Accelerator Breeder which would not involve either enrich-

ment or reprocessing. Finally, there is the assumption that the demonstration effect of going ahead with THORP will discourage rather than encourage others to do likewise.

Of course there is as yet no consensus of international expert opinion on these issues. For example, studies recently completed by the Swedish Atomic Forum suggest that spent fuel could be stored safely underground for at least 5,000 years in specially designed ceramic or copper canisters. This contrasts strongly with the views of BNFL and the UK Atomic Energy Authority, summarised in Para 8.14 of the Parker Report, which were that zircaloy fuel might be stored for up to 20 years and still remain suitable for handling and reprocessing, that it would be imprudent to store substantial quantities of stainless steel clad fuel in ponds for more 10 years, and that further evidence was required before present plans for early reprocessing could prudently be modified. Yet would anyone seriously expect BNFL—one of the parties to the Windscale dispute—to have come to a technical conclusion which was anything other than a vindication of its own practice?

The real truth about the storage/reprocessing debate is that there is a whole range of possibilities which is currently being reviewed within the International Fuel Cycle Evaluation Programme (INFCE), and that the attitude of each of the major parties depends most upon the strategic choices first made at the outset of its nuclear programme—in our case in favour of reprocessing to extract plutonium for our independent nuclear weapons programme in the late 1940's and early 1950's.

Then there remains legitimate doubt about the vitrification process, with which Mr Justice Parker said he was satisfied in Para 8.30 of his Report and which he predicted would succeed because it *had* to succeed. What a curious, but unfortunate, judicial syllogism! There is also persistent doubt surrounding the preferred method of final disposal for the highly

Nigel Forman is the author of *Towards a more Conservative Energy Policy*, Conservative Policy Centre, London 1977

active wastes, on which Mr Justice Parker's simple but bland comment in Para 8.31 was that "a final solution to the problems of disposal has not yet been found". Of course, he accepted in Para 8.32 that there should be no commitment to a *large* programme of nuclear fission power in this country until these and other waste disposal problems had been solved. It was readily apparent, however, that the Judge accepted the Government's view that the building of THORP does not necessarily imply such a commitment.

Yet this begs the question: what is a *large* nuclear programme in this context? The Government is now projecting 9.4 GW of net installed nuclear capacity in the UK in 1985 and the recent Energy Policy Green Paper envisaged perhaps 40 GW in the year 2000. Since THORP will not be fully on stream until the early 1990's and since by then we may well have some 20 GW of installed nuclear capacity in the UK meeting perhaps 30% of our electricity needs, at what point will the Government admit to having a *large* nuclear fission programme? The answer, it seems, is always just far enough into the future to be conveniently safe from falling foul of any inconvenient conditions which may be laid down by Royal Commissions or Judicial Inquiries.

Above all, there remains the serious doubt about Mr Justice Parker's excessively legalistic view of the vital non-proliferation issue. The fact is that it is political attitudes which are decisive in this matter and they are moving all the time towards a tougher more uncompromising position, not only within the US Administration, but also within the special forum of the Nuclear Suppliers' Group. The decision to go ahead with THORP thus flies in the face of American policy and is a calculated snub to most of the parties involved in the INFCE discussions. In this context, Mr Justice Parker was quite simply wrong in Para 6.23 of his Report when he argued that the building of THORP itself would not be counter to US policy.

The ultimate question, however, is whether or not there are such strong objections to reprocessing that the decision to build THORP should have been delayed or foregone. The fundamental argument for THORP is that we know how to do it and that if we had decided not to do it, others would have gone ahead, scooping the available foreign business in the process. It is also based on certain important energy policy assumptions which were not made very explicit in the Parker Report. These include the assumption that new reprocessing capacity of this kind will be a necessary but not a sufficient condition for series ordering of

fast reactors in this country—at any rate beyond the ninth such reactor. This in turn is based upon the assumption that we in the UK will have to depend heavily upon nuclear electricity for our energy needs in the 1990's and beyond, and that in such a situation we ought to minimise our dependence on foreign resources and technology.

In fact, Mr Justice Parker himself went further, making the assertion (Para 8.37) that "the only prudent course is to adopt a strategy which will give the greatest assurance that, no matter how the variables change, the energy needed to support *an acceptable society* can be provided" (the use of italics is mine). Not content with this, the Judge went on to argue in Para 8.40 that we should not divert available resources to energy savings and alternative energy sources "to an extent which would prejudice a large scale reliance on nuclear power".

Furthermore, it may be wondered why Mr Justice Parker and others seem to assume that considerations of resource and technology independence will appeal only to us in the UK; or why it is regarded as more or less inevitable that we shall have to reprocess a total of 6,000 tonnes of spent fuel from UK reactors alone by the year 2000; or why it is assumed that a refurbishing of Building 204/205 at the existing Windscale plant would not be capable of handling our own spent fuel requirements? The simple answer to such questions is that such assumptions were made in the Report because they constituted the bedrock of BNFL's evidence to Mr Justice Parker at the Inquiry and because that was the evidence which he chose to accept in preference to that of the objectors.

However, it should be asked whether the Judge was wise to have based his crucial assumptions so heavily upon BNFL's so-called realistic forecasts, when their origin was in fact none other than the planners in the Department of Energy and the Atomic Energy Authority. And was it really wise for the Judge to have dismissed the alternative views of reprocessing on the grounds that they were "of recent origin" (Para 3.1) or that they are sustained by public anxieties which he chose to describe as "needless", "without foundation" and even "irrational and misplaced" (Para 2.1)? This is hardly the sort of language that people expected to find in an impartial, judicial Report.

The Windscale decision ought not to have been seen as a choice between the risks of reprocessing and the risks of energy shortage by relying solely on non-nuclear means, which was how Mr Tony Benn expressed it in winding up the Debate on 15 May. Many of those who retain serious doubts about

THORP are not advocating the elimination of what the Secretary of State described as the "nuclear component" in our energy mix. It would be possible to have a nuclear component without THORP and it would even be possible to have it without reprocessing at all. False polarities in the debate have not assisted clarity of thought or decision.

The Government's case for proceeding with THORP was essentially:

- that the nuclear component is necessary for UK energy policy;
- that THORP is necessary for that nuclear component to develop properly; and
- that the objections raised at the Inquiry did not justify postponement or cancellation.

It is clearly possible to accept the first argument without necessarily accepting the second and third.

In the first place, nothing would really have been lost by postponing a decision until the outcome of the INFCE discussions is known in about 18 months time. Indeed, BNFL itself is on record in Para 8.6 of the Parker Report to the effect that a start on THORP could have been delayed for up to five years without any irreparable harm being done.

Secondly, since the decision involved a project which will not be fully operational until the early 1990's and since the Japanese spent fuel, for example, will have to be stored in any case for some years before it is put through THORP, there would have been little or nothing to lose from spending a bit more time in serious international investigation of all the possible alternative fuel cycles, making further vigorous efforts to stuff the genie of nuclear proliferation back into a safer bottle, and giving further sober consideration to the new more conservative energy forecasts upon which all our future plans should now be based. After all, even the greatest energy experts are still relatively low on the learning curve about the full potential of safer, cheaper, alternative energy futures.

Of course, a counsel of delay would be irresponsible if it were not based on the assumption that the best possible use would have been made of the breathing space thus gained to investigate all the available alternatives to THORP, and that further rigorous conditions would have been imposed on the development had it been given the go-ahead in the end. However, from a responsible and even ethical point of view a decision to delay would have provided a rare opportunity in the developed world to show that we are capable of moving from the politics of prescription to the politics of proscription. That would have been a signal of hope for mankind. □

correspondence

SO₂ emission in the UK

SIR,—The final report resulting from the study of transboundary air pollution in Western Europe by the Organisation for European Co-operation and Development was summarised in *Nature* (268, 92; 1977). The study was instituted because of the reported ecological damage in Scandinavia which was linked with the doubling of emissions of sulphur dioxide in Europe (figure 1) arising from the combustion of fossil fuels. The UK has often been criticised as the major contributor to transboundary air pollution because it discharges more sulphur dioxide into the atmosphere than any

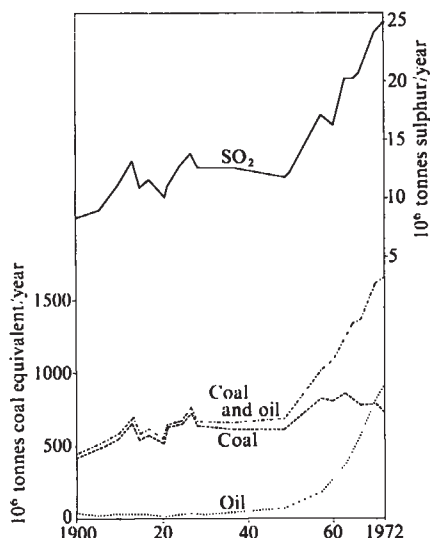


Fig. 1 Fossil fuel consumption and estimated anthropogenic SO₂ emissions in Europe 1900–1972 (Source: After Fjeld Nilu Teknisk Notetnu 1/76 (1976)).

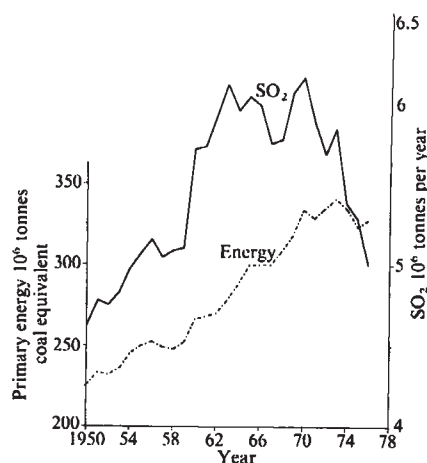


Fig. 2 Primary energy consumption and SO₂ emissions (Source: Warren Spring Laboratory and UK Energy Statistics) (By permission of the Controller of HMSO).

other country in Western Europe. It is, however, interesting that the trends of emissions in the UK differ from those in most other countries and, as figure 2 shows, over the period 1950–75 when emissions in Europe roughly double those from the UK reached a maximum during the 1960s and declined thereafter; they are now approaching the 1950 level.

The rise and fall of sulphur dioxide emissions since 1950 is not a consequence of a reduction in demand for energy. As figure 2 shows primary energy consumption has been rising steadily over the period except for a drop in the mid-1970s at a time of economic recession. Thus by the use of cleaner fuels such as natural gas, low sulphur oil and nuclear energy, the UK has substantially reduced the emissions of SO₂ to air. How far this is of benefit to other countries remains to be seen: it is certainly one of the factors which has contributed to a reduction of more than 50% in ground level concentrations of sulphur dioxide in urban areas in the UK over the last 25 years although the concentration in urban areas cannot be directly related to total emissions.

To predict future trends is always hazardous. But even at an optimistic rate of growth for energy within the UK the total emissions of sulphur dioxide are likely to fall still further within the next few years.

Yours faithfully,
L. E. REED

Department of the Environment,
London, UK

Indirect research costs

SIR,—Many research scientists in the United States will applaud the recent Office of Management and Budget rules on indirect research costs. Since the total funding for each research area is fixed by Congress, each dollar that a university is able to claim in "indirect costs" is a dollar less that is available for the direct cost of research. The present system of figuring such costs is chaotic. The amount of money that a university can claim in indirect costs is limited only by the ingenuity of its accountants and, as might be expected, accountants at the elite private universities seem to be the most ingenious. Consequently indirect cost rates can vary by a factor of two or three between institutions, rewarding those that have allowed their costs to grow uncontrollably and penalising those that are most efficient. As a result of a system that allows universities to claim that "indirect costs are whatever we say they are", there is virtually no money available to support the direct costs of research by younger investigators and investigators at less prestigious institutions.

The OMB regulations are long overdue, but they do not go far enough. What is needed is an absolute limit on indirect costs, and some individuals have claimed

that 25% to 30% of direct costs would be a reasonable figure. Here in Canada, the National Research Council and the Medical Research Council will not reimburse universities for any indirect costs and yet research still gets done.

Yours faithfully,

ROBERT JOEL YAES
Memorial University of Newfoundland,
Canada

Perutz not retired

SIR,—Contrary to widespread belief, I have not yet retired. I shall remain chairman of this laboratory until Dr Sydney Brenner takes over on 1 October 1979. The laboratory's governing board has kindly invited me to continue working here after that date. I intend to do so and shall be pleased to receive graduate students and postdoctoral workers who want to come to work with me.

Yours faithfully,

M. F. PERUTZ
MRC Laboratory of Molecular Biology,
Hills Road, Cambridge, UK

Allez les verts

SIR,—Following an earlier article entitled 'Ecologists lose in French elections' (30 March, page 393), M Porchez explains in his letter (4 May, page 8) that there has never been a particular ecological group known as 'les verts' (the green) but that it is a name popularly applied in France to all ecologists. Others would doubtless claim prior use of this appellation, but the exhortation "Allez les verts" displayed ubiquitously in France on car windscreen stickers probably reflects quite simply an unusually high proportion of ecologists in St. Etienne's football team.

Yours faithfully,

G. H. COOPER
Salisbury, Wiltshire, UK

Portuguese Africa forgotten

SIR,—I was delighted to read (20 April, page vi-vii) of 'The discoveries of Africa 1493 B.C.—1978 A.D.' that Swissair obligingly made public.

The advertisement quickly shattered to dust all the efforts made by the Portuguese in the XVth and XVIth centuries to add "new worlds to the world," as Camoes, whom Ezra Pound calls "the Rubens of verse", nicely puts it.

It is neither imperial nostalgia nor the preservation of a national *epos* that prompts me to write this, but the feeling that even an advertisement should be free of bias.

Yours faithfully,

ANTÓNIO CASTRO FEIJÓ
Lisbon, Portugal

news and views

Cell surface marker for malignancy

Kenneth M. Yamada

MANY tantalising hints suggest that changes in the cell surface have important roles in malignancy. Over the past decade, studies with cells in tissue culture have identified many alterations in the plasma membrane after malignant transformation *in vitro* by oncogenic viruses and carcinogens. These alterations include increased rates of nutrient transport, increased agglutinability of cells by certain plant lectins, decreased quantities of an adhesive glycoprotein of high molecular weight, and structural changes in glycolipids and glycopeptides. However, none of these cell surface parameters alone seems to predict with certainty whether cells will form tumours after injection into test animals.

Certain growth characteristics of transformed cells, particularly their capacity to continue growing after suspension in semi-solid medium, are currently considered markers of malignant transformation. Recently, a novel approach involving hybrids between normal and malignant cells has been utilised to re-examine the effectiveness of *in vitro* growth characteristics as markers for transformation. Although these markers are not found to be reliable, a new membrane marker for malignancy has been identified.

What criteria would establish an ideal marker for malignancy? First, the alteration must regularly accompany malignancy. The alteration should be present in all cell lines that can produce tumours when injected into test animals and in tumours *in vivo*, and it should be absent from all non-tumorigenic cell lines and from normal tissues.

Second, the alteration must not result solely from differences in growth characteristics of normal and cancerous cells. For example, if rapidly dividing normal cells also display the

alteration, it is not a characteristic of malignancy.

Third, the marker should be genetically linked to malignancy. Experimental selection for or against the marker itself should also select for or against malignancy, and *vice versa*. For example, a procedure that selects for cells that have lost the marker should also select for non-malignant cells.

Fourth, the alteration should ideally be an essential step in the process of malignancy. Although this criterion is theoretically not necessary, it would provide the most convincing evidence for a marker.

H. Harris, G. Klein, and others had found that cell hybrids resulting from the fusion of a wide variety of normal and malignant cells are usually incapable of forming tumours in test animals (Harris *et al.* *Nature* **223**, 363; 1969; Stanbridge *Nature* **260**, 17; 1976). When these hybrids are maintained in cell culture, they often eventually produce sublines of cells that are once again tumorigenic. These series of lines provide sets of related tumorigenic and non-tumorigenic cells for evaluating markers of malignancy.

When these hybrids are examined using the conventional *in vitro* growth markers for transformation, problems arise. For example, the capacity of cells to grow in soft agar and in low concentrations of serum no longer reliably predicts whether certain hybrids will be tumorigenic (Straus, Jonasson & Harris *J. Cell Sci.* **25**, 73; 1977; Stanbridge & Wilkinson *Proc. natn. Acad. Sci. U.S.A.* **75**, 1466; 1978).

In search of a more reliable marker, Bramwell and Harris (*Proc. Roy. Soc. B* **201**, 87; 1978) have compared the proteins and glycoproteins of plasma membranes from a variety of normal, tumour and hybrid cells. They find that the only reproducible change is associated with a glycoprotein of 100,000 daltons. In malignant cells, this protein binds substantially larger quantities of the lectin concanavalin A

and less wheat germ agglutinin (WGA) than the same protein from non-tumorigenic cells.

Specifically, these authors separated proteins by gel electrophoresis in sodium dodecyl sulphate, then labelled the glycoprotein bands with concanavalin A or WGA tagged with ¹²⁵I and determined the percentage of total binding present in the 100 K band in normal compared with tumorigenic cells. Since concanavalin A binds to mannose and glucose, while WGA binds to N-acetyl glucosamine, their findings suggest a specific abnormality in the glycosylation of the 100 K protein.

This abnormality was present in a variety of malignant cells including carcinoma, sarcoma, lymphoma, and melanoma cells, and it was absent from normal and hybrid cell lines that were non-tumorigenic. Hybrid clones that had re-expressed malignancy again displayed this alteration. The alteration in concanavalin A binding was usually even more marked if glycoproteins were extracted directly from tumours.

The alteration does not seem to be solely secondary to differences in growth characteristics, since similar lectin binding to the 100 K protein is found in growing compared with stationary cultures of normal cells.

To take advantage of the altered glycosylation of tumorigenic cells, Bramwell and Harris tested lectins for selective toxicity to tumour cells. They find that WGA is toxic to a number of malignant lines. It selects for cells that display a more normal concanavalin A binding pattern, and that are less tumorigenic. Tumours that eventually arise from some clones once again displayed abnormal concanavalin A binding. Although this approach may not involve a direct selection against the marker, it does provide further genetic evidence for linkage between the marker and malignancy.

Before this marker will be generally accepted, similar alterations in 100 K

Kenneth Yamada is an Investigator and Senior Surgeon in the Laboratory of Molecular Biology, National Cancer Institute.

protein glycosylation should be demonstrated in more widely utilised cell lines transformed *in vitro* by viruses. The most convincing demonstration would be with temperature-sensitive viral mutants in which the transformed state can be expressed or suppressed rapidly by altering temperature. The only published study of lectin-binding to proteins of viral transformants may show similar alterations in WGA binding, but quantitation was not performed (Burridge *Proc. natn. Acad. Sci. U.S.A.* **73**, 4457; 1976).

Other important future work obviously includes identifying the structural basis of the altered lectin binding of the 100 K protein, and investigating whether these changes are directly responsible for any aspect of malignancy.

Is the 100 K protein a known plasma membrane protein? The authors speculate that it is the insulin receptor. However, it could be a

recently isolated membrane protein of 97,000 daltons that is induced in some transformed cells secondary to glucose deprivation (Shiu, Pouyssegur, & Pastan *Proc. natn. Acad. Sci. U.S.A.* **74**, 3840; 1977; Pouyssegur & Yamada *Cell* **13**, 139; 1978).

The possible benefits from the unequivocal identification and isolation of a general membrane marker for malignancy might include simpler diagnosis of the disease, immunotherapy directed against the marker, and a clue to the mechanisms of malignancy. However, at present the evidence for this marker is not unequivocal, and the history of other proposed membrane markers for malignancy suggests that exceptions may be found. Regardless of the final verdict, studies of the changes in glycosylation of this 100,000 dalton glycoprotein should provide useful information about the process of malignancy. □

Cosmic heresy?

from P. C. W. Davies

SINCE burning at the stake is now out of vogue, it will be interesting to see how the Establishment responds to a radical new model of the Universe proposed by George Ellis. For he does not merely tinker with the currently fashionable big-bang cosmic scenario, he goes the whole way and abandons the entire conceptual and philosophical foundation of modern cosmology.

Returning to a pre-Hubble, some would say pre-Copernican, picture of the Universe, he describes in the current issue of *General Relativity and Gravitation* (**9**, 87; 1978) a model cosmos with the startling feature of being static. Since the discovery in the late 1920s by Edwin Hubble of a systematic redshift in the light from distant galaxies towards the red end of the colour spectrum, it has been universally accepted that this phenomenon is due to a more or less uniform overall expansion of the Universe, resulting in a general, rapid, recession of all galaxies away from one another.

It has always been realised, however, that a redshift of light can have another cause—gravity. When light climbs away from a massive gravitating object it loses energy, a depletion which shows up as a loss of frequency (colour shift). This explanation for the galactic redshift has always been ruled out because it is asymmetric: light falling towards a mass acquires a blueshift. As we see only redshifts whichever direction we look in the sky, the

only way in which this could be consistent with a gravitational explanation is if the Earth is situated at the centre of an inhomogeneous Universe. This is precisely what Ellis suggests.

Actually there is a subtlety. Imagine a static blob made up of galaxies, with a high concentration near the centre and a more sparsely populated periphery. An observer at the centre would see blueshifts as the light from the galaxies near the edge fell inwards. However, the possibility of curved space provided by Einstein's theory of relativity enables the 'edge' to bend around on itself and form another centre, or rather a sort of 'anticentre' in a higher-dimensional version of the antipodes. At the anticentre an observer would see only redshifts. It is here that Ellis conjectures the Earth may reside.

The idea of a privileged cosmic location for man has been anathema to scientists ever since Copernicus showed the Earth is not at the centre of the Universe but revolves round the Sun. All subsequent astronomical discovery has appeared to confirm the fact that we inhabit an unremarkable region of the cosmos, and that as successively larger regions are surveyed by our telescopes, they sample what always appears to be a typical portion of the whole. Now Ellis is suggesting that this assumption is more a philosophical prejudice than a result of hard scientific evidence. In his new model, the Earth does indeed reside near the ('anti')centre, but for biological reasons rather than theological ones. He argues that the physical conditions

near the centre, particularly the prevailing temperature, are especially favourable for life to evolve, so it is no surprise that we find ourselves in such a special location. (After all, we are not surprised to be living on the surface of a planet, even though most of the Universe is empty space.)

But that is not all. The other centre is not merely more densely populated with galaxies: the concentration of matter there is so great that the curvature of space rises without limit. Indeed, our cosmic antipodes is inhabited by that ultimate nightmare of physics—a naked singularity. This is a region where space comes to an end and causality breaks down. According to Ellis, the naked singularity is what astronomers have up to now mistaken for the big bang—a sudden cosmic creation event, 15 billion years ago. In the new model, the singularity is not a past boundary of time, but a permanent feature, marking a boundary of space.

Not only does the Ellis Universe contain this bizarre structure, but the singularity actually plays an indispensable role as a sort of cosmic recycling mechanism. As galaxies burn out, so their material falls towards it, to be replaced by fresh hydrogen spewed outwards. In this way, the singularity continually rejuvenates the Universe, endowing it with unlimited longevity.

The idea is reminiscent of the late lamented steady-state theory, except there is no necessity here for the continual creation of more and more matter to 'top up' the Universe as it expands. Moreover, the Ellis model does not suffer from the major defect of the steady-state: its inability to account for the cosmic background heat radiation, widely presumed to be the last fading glow of the primaeval fire. This radiation, first detected in the mid-1960s, has a temperature of a mere 2.7 K, and apparently bathes the whole Universe. It is considered to be the best evidence that there really was a hot big bang. Now Ellis conjectures that this thermal radiation comes not from any primaeval phase, but from the intensely hot material which shrouds the singularity.

The essence of Ellis's argument is that the currently available data about the structure and evolution of the Universe on a large scale are quite consistent with the picture of a static inhomogeneous Universe observed from a very special location. One major difficulty seems to be stability. A good way of viewing the model is to think of the fiery singularity as located (appropriately enough!) at the 'bottom' of the Universe, while our own elevated location is at the 'top'. The question is, could we remain perched precariously over such a great

mass concentration without the whole Universe collapsing under its own gravity down into the singularity, like a gigantic house of cards?

A further problem concerns the precise details of the recycling mechanism, whereby the heavy elements (ashes of the stars) are flushed down towards the singularity and the replacement light elements, such as hydrogen, which are used to build up new generations of stars, drift up towards us.

Needless to say there are also a number of philosophical problems. Naked singularities are usually regarded as a serious disease in physics, something to be avoided at all costs. Most relativists support Roger Penrose's idea of a cosmic censor who always clothes singularities inside black holes, where they cannot influence the outside world. In the Ellis model the singularity—by definition a lawless and unpredictable creature—is contrived to drive, ultimately, all the organised activity in the Universe.

There is also the curious problem of why, if the Universe is infinitely old and life is concentrated in our particular corner of the cosmos, it is not inhabited by technological communities of unlimited age. Is technology recycled too? In a cryptic footnote to his paper Ellis comments that it may be desirable to find a compromise model Universe, incorporating features both of the standard expanding model and his own new ideas.

In spite of all the difficulties, this remarkable new slant on the cosmic structure and organisation serves at least as a reminder that the conceptual basis of modern cosmology can be widened. Perhaps the Copernican revolution is over? □

Superfluid ^3He at Manchester

from P. V. E. McClintock

Now that the superfluid phases of liquid ^3He (discovered at Cornell University in 1972) are no longer quite such a novelty, it seems timely to pause, take stock of what has been achieved and consider how best to tackle the substantial problems still remaining. It had always been clear that a number of the early experiments paid insufficient attention to charac-

terising the textures exhibited by the (highly anisotropic) superfluids; and it was entirely fitting, therefore, that a recent meeting on superfluid ^3He at Manchester* should have been notable for people's concern about how to predict and control the textures taken up by the liquid under different sets of experimental conditions.

D. Bailin (University of Sussex) described the ways in which topology could contribute to an understanding of the textures and transitions between them. Although he sought to emphasise that because one does not feed very much information into a topological analysis one cannot reasonably expect to get much out again in the way of quantitative conclusions, it became clear in the course of his talk that a good deal of insight may nonetheless be gained by this approach.

One method of controlling the texture experimentally lies in careful choice of the shapes of the surfaces with which the liquid comes in contact: it was reported that the Sussex NMR experiments were being conducted on liquid held in fine tubes $1\mu\text{m}$ in diameter; whereas, at Orsay, the liquid was contained within a stack of 0.4 mm Mylar plates. Other factors affecting the texture were the applied magnetic field, which was easy to control, and heat flow, which was not. The effect of a flow of heat was expected to be quite complicated and it could even give rise to 'dynamical textures', that is, textures which vary systematically with time, according to J. R. Hook (Manchester University). It seemed very likely that the long lived periodic motions observed earlier by J. C. Wheatley and his coworkers at La Jolla were a consequence of this effect.

J. D. Reppy (Cornell University) described recent high precision experimental results obtained at Cornell using what amounts to a modified Andronikoshvili apparatus: an inverted torsion pendulum whose hollow interior could be filled with liquid ^3He . By measuring changes in the characteristic frequency and damping coefficient attributable to the ^3He , values could be deduced for the average viscosity and superfluid density, and interesting comparisons could then be made with viscosities measured by a completely different method employing a vibrating wire.

Summing up and pointing towards possible future developments, A. J. Leggett (Sussex University) discussed some of the puzzles still being posed by superfluid ^3He . Coping effectively with textures presented severe theoretical difficulties, and he enthusiastically

endorsed an earlier remark by Hall to the effect that hoping to predict textures from the equations of superfluid hydrodynamics was almost as ambitious as expecting to be able to predict the weather by solving the Navier-Stokes equations! An experimental approach was best in the first instance, and he went on to discuss the techniques, such as ultrasound, already available as well as an interesting, and as yet unproven, idea involving possible reflection of quasiparticles when they enter regions where the energy gap, relative to their direction of motion, is decreasing. Other important but unresolved puzzles included: the absence of the electric field effect (electric fields had been expected to orientate the l vector, but experimentally they apparently failed to do so); the magnitude of the A-phase liquid's intrinsic angular momentum (a bucket of initially normal liquid ^3He should start to rotate when cooled into the superfluid A-phase, but how fast?); anomalous differences between static and NMR measurements of the B-phase magnetic susceptibility; and the range of validity of the (so far, remarkably successful) 'giant diatomic molecule' picture of the superfluids. The latter model has, of course, recently received something of a boost through Leggett's own successful use of it for predicting, correctly, that the A-phase would be weakly ferromagnetic. □

More puzzles about the early Solar System

from M. Edmunds

THE determination of the relative abundances of isotopes in meteorites continues to produce unexpected results. A particularly copious and fruitful source of this early Solar System material has been the meteorite from Allende in Mexico. The relatively high abundance of ^{26}Mg , the decay product of ^{26}Al , in some aluminium-rich minerals in this meteorite has already prompted suggestions that heavy elements freshly made in a supernova explosion were incorporated into solid material during the formation of the Solar System (see *News and Views* 267, 393; 1977). Anomalies in the isotopic

P. V. E. McClintock is a member of the Department of Physics at the University of Lancaster.

*Held on 28 April.

M. Edmunds is in the Department of Applied Mathematics and Astronomy at University College, Cardiff.

composition of volatile elements like O and Xe have been known for some time, and now work by G. J. Wasserburg and his colleagues (*Astrophys. J. Lett.* **220**, L15, L21; 1978) points to the first definite anomalies in refractory elements other than Mg.

The abundance anomalies exist in small distinct regions (or 'inclusions') in the meteorite, and inclusions showing two completely different types of anomaly have been found. The first type shows excess ^{26}Mg which, as already mentioned, implies contribution of freshly made supernova products. These inclusions show perfectly normal (that is terrestrial) abundance ratios of the three refractory elements analysed—Ca, Ba and Nd. The second type of inclusion appears to be highly chemically fractionated and shows anomalies in Mg isotope ratios which can be interpreted as either a deficiency in ^{26}Mg and ^{24}Mg , or an enhancement of ^{26}Mg . Only two of this second type of inclusion have been analysed in detail, but one of them shows excesses of ^{42}Ca , ^{138}Ba , ^{137}Ba , ^{145}Nd and a large excess of ^{44}Ca , together with deficiencies of four Nd isotopes. The other inclusion of this type shows a deficiency of ^{44}Ca and ^{138}Ba , although Nd and the remaining isotopes of Ca, Ba are normal.

The real problem in interpreting these anomalies arises because the isotopes involved are believed to be made in different nuclear processes. Although all these processes could occur during the extreme physical conditions accompanying the supernova explosion of a massive star, the processes take place in different regions of the exploding star. In the canonical model, shells (or 'zones') of material at different radii undergo different reaction chains. For example, ^{42}Ca is thought to result from a series of reactions starting with oxygen, while ^{44}Ca is believed to result from reactions in another zone. The Ba and Nd anomalies could be explained by addition of material from yet another zone where intense neutron fluxes give rise to the so-called 'rapid' neutron capture chain of reactions. The occurrence of peculiar ratios of isotopes from different nuclear sources within a single inclusion therefore implies a contribution from several sources to each inclusion. The variation of these anomalies between different inclusions implies considerable variation in the relative contribution of each source to the different parts of the early Solar System nebula of gas and dust out of which the solid material formed. If the excess ^{26}Mg anomalies are accepted as good evidence for a supernova explosion just before Solar System formation, then the implication is that

material thrown out of each supernova zone was very inhomogeneously distributed into the nebula. The inhomogeneities must also have been incorporated into solid material before mixing processes could even out the isotopic (and presumably chemical) composition of the nebula. It is interesting that the isotopic anomalies are the same for the different minerals contained in each inclusion. This suggests that the inclusions as now seen must have been formed (or passed through a stage) where the contributions from each source were melted or vaporised and then recondensed or solidified to distribute the isotopes uniformly between each mineral.

The identification of the new refractory isotope anomalies is not yet completely unambiguous, since it depends on which isotope of an element is used as a standard against which to normalise the abundances of its other isotopes. Exactly how much fractionation has occurred remains uncertain, although the authors speculate that some of the apparent fractionation may in fact be just due to unequal contributions from the different nuclear sources. Results on the rare-earth elements are eagerly awaited, since the similarity of their chemical properties will reduce fractionation effects.

Isotopic anomalies undoubtedly exist, the range of elements showing them now includes O, Ne, Mg, Ca, Kr, Xe, Ba, Nd, and others may well follow. The chemical and nuclear inhomogeneity implied by these latest results may considerably complicate the building of models of the formation of the Solar System. □

Some recent results in X-ray astronomy

from J. J. Quenby

FURTHER discussion of results arising from Britain's long running space success, the Ariel 5 X-ray satellite, provided the basis of a recent Royal Society meeting*. Launched in October 1974, observations are expected to continue until September 1979 at least.

Among the new reports on galactic sources, P. Sanford (Mullard Space Science Laboratory (MSSL)) mentioned an interesting, slowly rotating X-ray pulsar which apparently exhibits a double periodicity. This source,

4U1145-61, seems to show both a 4.95 m and 4.86 m regularity in its time behaviour. Explanation of this unique Ariel 5 observation may lie in the fact that one period is due to the motion of a neutron star magnetosphere where the X-ray generation takes place while the other arises in the interaction of these X rays with a rotating disk of material accreting from a companion star.

The one US participant in the meeting representing experimenters from the HEAO-1 heavy explorer program was H. C. Friedman of NRL who provided many examples of the more sensitive observations which his group is beginning to achieve with a large area sky survey instrument. These included a demonstration that the fast fluctuations seen in Cyg X-1, the well-known black hole candidate X-ray source, disappear on a millisecond time scale although there is much variability at 40 ms and slower scales. This result seems to fit with an Imperial College/MSSL/Birmingham Ariel 5 study of Ser X-1, a rather similar object, which finds that the power in such fluctuations at lower frequencies is inversely proportional to frequency. The physical cause may lie in the growth of various instabilities inherent in the very hot and tenuous inner disk of accreting material (see Shakura & Sunyaev *Mon. Not. Roy. Astr. Soc.* **175**, 613; 1976).

Short period binary star systems, consisting of a late type star and a white dwarf and which characteristically are seen as optical novae, are becoming established as X-ray sources. Leicester results reported by M. G. Watson on SS Cygni indicate the coincidence of optical and X-ray flares in such a system, but with the X rays suppressed just at the peak of the optical emission. Some aspects of a shock heating mechanism for accreting material working in conjunction with a high white dwarf magnetic field, as put forward by Fabian *et al.* (*Mon. Not. Roy. Astr. Soc.* **175**, 43; 1976), fit in well with the observation. However, G. T. Bath (University of Oxford) argued that optical data required the presence of a conventional accretion disk and hard X-ray results on a rather similar source (see *Nature* **272**, 38; 1978) seem to require something more than the predicted thermal bremsstrahlung emission from such a mechanism.

Improved spatial resolution in a measurement of the X-ray emission from the expanding shell of a supernova remnant, the Cygnus Loop, has been achieved in a joint MIT/Leicester rocket flight with an X-ray imaging system. X rays coming preferentially from shock heating at positions where the shell interacts with interstellar

*Held on 25-26 April.

matter were clearly delineated in the results presented. Again on supernova remnants (SNRs), Murdin, Clark and J. L. Culhane, an AAT/MSSL team, have exploited the fact that optical 'coronal lines', from ^{56}Fe in particular, indicate temperatures in a plasma of a few 10^8 K , just sufficient to provide significant soft X-ray emission. They have recently measured these lines in the SNR N49 in the Larger Magellanic Cloud and are able to predict X-ray emission in the 0.28 to 8 keV range at a flux of a few $10^{-12}\text{ erg cm}^{-2}\text{ s}^{-1}$. Confirmation of this expectation is awaited.

Detailed mapping of the galactic centre region X-ray sources was reported by G. K. Skinner (University of Birmingham) using both Ariel 5 and new rocket data, the latter accurate to 4 arc min in one dimension. One surprise was that three short X-ray bursts arrived during the few minute rocket exposure. An extended 'steady' source, close to the galactic centre and designated by the Birmingham Group as GX+0.2, -1.2, seemed to be one of the X-ray burst sources.

The 13 discrete galactic γ -ray sources seen by the COS-B satellite above 50 MeV (Hermesen *et al.* *Nature* 269, 494; 1977) have recently received attention from the Imperial College group. In most cases Ariel 5 and other satellite data are shown to be consistent with an E^{-2} photon spectrum. Extrapolating this relation to the whole of the galactic disk, instead of the limited area where the COS-B analysis was performed, implies that on average all the COS-B galactic disk emission could be of point source origin. Put in another way, it is at present very difficult to distinguish those γ -rays resulting from the interaction of cosmic rays with interstellar material from those due to processes in what are basically hard X-ray stars. As A. W. Wolfendale (University of Durham) pointed out, there is a danger that we subtract so much due to point sources in the galactic anti-centre direction in particular that there is no room remaining for a finite cosmic ray flux beyond our position in the Galaxy.

Turning finally to the realm of extragalactic objects, K. Pounds, whose Leicester group has already done much in the identification of Type I Seyfert galaxies with X-ray emitters, announced that five X-ray objects correspond to active emission line galaxies. These galaxies have spectral line widths corresponding to velocities of roughly 400 km s^{-1} , rather less than those of Seyferts. The absolute luminosities in the X-ray and radio wavebands are also

rather less than the Type I Seyfert examples identified, but nevertheless non-thermal processes are still required to produce the overall electromagnetic spectrum. It is interesting that three of the objects were discovered first in the X-ray band and only subsequently at optical wavelengths.

Concerning clusters of galaxies, Culhane discussed evidence indicating that some active galaxy members shine independently at temperatures rather different from that of the general, hot intergalactic gas which is usually thought to be responsible for the main component of the X-ray emission from clusters in the 1–10 keV energy range. Davidson's (MSSL) recent analysis of M87 in the Virgo cluster provides a possible example of this idea. This

shows the peak in the 1–5 keV flux located on M87 while the peak in the 8–14 keV flux is shifted about a degree and located close to M86. In discussion A. Fabian (Institute of Astronomy, Cambridge) maintained that the effect of an exceptional gravitational potential well on the general intergalactic hot gas distribution was sufficient to explain the observed spatial variations in X-ray temperature.

This brief account of the latest results from Ariel 5 indicates the continued UK contribution to this field of research. It is the fervent hope of all the experimenters involved that a future (1980s) opportunity to instrument with improved techniques a further UK X-ray satellite will present itself. □

Cuticle under attack

from J. F. V. Vincent

MUCH interest in insect cuticle as a vulnerable system for attack by pesticides has been generated by the discovery of Dimilin (made by Philips-Duphar of Holland). This pesticide inhibits the synthesis of chitin, the cellulose-like polysaccharide which forms the fibrous framework of arthropod cuticles, and weakens the cuticle so that the insect is unable to moult. At a recent workshop on the biochemistry and ultrastructure of insect cuticle* D. H. Deul (Philips-Duphar) showed that Dimilin acts by inhibiting the chitin synthetase which sits at the epidermis-cuticle interface. Inhibition of chitin synthesis affects the deposition of proteins within the cuticular matrix. L. Clarke (University of Reading) showed that the matrix of soft cuticles is unable to stabilise additional protein in the absence of chitin—in such circumstances the cuticle does not grow. Cuticles destined to be cross-linked and become hard are not so affected and continue to stabilise protein in the absence of chitin. Presumably the proteins have some other means of stabilising themselves within the matrix, probably through hydrophobic interactions. Electron micrographs of cuticle affected by Dimilin (R. Ker, University of Oxford) confirm that some cuticles (for example, locust tibia) can deposit protein in the absence of chitin and that some (for example the extensor tibiae tendon of the locust) cannot. The normal deposition zone immediately outside the epidermal cells is disrupted when Dimilin is present and globules of material, presumably

protein, appear instead. The effects are reversible if Dimilin is withdrawn.

The ability of proteins to stabilise within the cuticle clearly shows that their aggregation behaviour is important in the mechanical stability of the cuticle. J. E. Hillerton (University of Reading) reported that the extensible cuticle of *Rhodnius* larvae contains a protein aggregate made up of six subunits of molecular weights 30,000, 33,000 and 60,000 when separated on SDS gels, but which behaves as a single unit in a simple buffer gel. The aggregate has a ring structure when seen in the electron microscope and can form higher complexes. In aqueous buffers the aggregate has a negative temperature solubility coefficient like tubulin and spectrin. This unusual protein may be important in controlling the onset of cuticle plasticity when *Rhodnius* feeds and the cuticle stretches. J. F. V. Vincent (University of Reading) showed that there are at least 31 different subunits in the extensible intersegmental membrane cuticle of the female locust. When subjected to isoelectric focusing in a urea gradient these proteins show shifts in isoelectric point indicating changes in aggregation. Such aggregation is necessary to generate the long polymer chains (molecular weight about 1,000,000) required to account for the mechanical properties of this highly extensible membrane. Evidence suggesting that resilin, another elastomeric component of cuticle, is composed of at least two subunits of

J. J. Quenby is in the Department of Physics, Imperial College of Science and Technology, London.

*Held on 11th May at the Royal Entomological Society, London.

Julian Vincent works in the Biomechanics Group, Department of Zoology, University of Reading.

molecular weights 100,000 and 18,000 was presented by L. Routledge (University of Cambridge).

The focus of biochemical interest is still the mechanisms of sclerotisation (cross-linking) in the insect cuticle, a subject bedevilled by much misguided and inconclusive research. P. C. J. Brunet (University of Oxford) pointed out that we really know little more now than we did after Pryor published his first observations on the sclerotisation of the cockroach ootheca in 1940. Brunet's message was, essentially, go back to Pryor's work and start again! S. O. Andersen (University of Copenhagen), echoing one of Pryor's ideas, proposed that the sclerotising quinones form bulky complexes in a manner very similar to lignification in plants. The novelty of his model is that the hydroxyls of one aromatic ring link across a carbon-carbon double bond in the side chain of another aromatic ring. He is now chasing the enzyme responsible. A quantum jump in the investigation of sclerotisation which avoids the difficulties inherent in

destructive biochemistry has been made by R. N. Pau (ARC Unit of Invertebrate Chemistry and Physiology, University of Sussex), who has started looking at cross-linking *in situ* using NMR techniques. He can follow the course of sclerotisation in the cockroach ootheca and detect changes in bonding of the sclerotising phenols and in specific amino acids of the protein. His preliminary results show great promise in analysing the reactions as they occur.

However, despite the fact that biophysicists and biochemists are taking cuticle apart they cannot yet say how the components interact to form a multipurpose exoskeleton. S. E. Reynolds (University of Bath) described the effects of eclosion hormone and bursicon on cuticle mechanics, but no-one could explain what happens within the cuticle to produce these effects. We need to know more about the mechanics of cuticle and their biochemical basis. That such results could have practical implications is shown by the success of Dimilin. □

reaction that was about 200 times smaller than that observed experimentally.

The form of the α -nucleus potential was obtained by folding the known α -nucleon potential with the density distribution of ^{12}C , and this gives a wavefunction with a root mean square (RMS) radius of 3.03 fm, which is somewhat larger than the measured ^{16}O RMS radius of 2.65 fm. Nevertheless, the calculated spectroscopic factor (a number measuring the quantum-mechanical overlap between the ^{16}O wavefunction and the product of the ^{12}C and α -particle wavefunctions) is 0.23, compared with a value of 40.3 obtained from the experimental data. To obtain such a value, it is necessary to increase the radius parameter of the potential from $1.09 \times 12^{1/3}$ to $2.52 \times 12^{1/3}$ fm, corresponding to a RMS radius of 4.37 fm. This α -particle wavefunction peaks very far out in the nuclear surface, indicating the importance of α -clustering for such reactions.

The α -clustering indicated by this work could either be already present in the target nucleus before the interaction, or it could be produced by the interaction. This latter possibility could arise if there is a strong coupling between the entrance channel and one or more excited states with large probabilities for decay by α emission. Coupled channels calculations of this type of process would be of great interest. □

Alpha-particle clustering in light nuclei

from P. E. Hodgson

ONE of the most interesting questions about nuclear structure is the extent to which the nucleons tend to group together in clusters, particularly α -particle clusters, as these are the most probable because of their high symmetry and stability. It is conjectured that these clusters are continually forming and reforming inside the nucleus as the nucleons interact with each other. This simple classical picture must be expressed quantum-mechanically in terms of the probability of the overlap of the wavefunctions of the four constituent nucleons, two protons and two neutrons. There is then always a small overlap even when classically we would say that there is no α -clustering, so we must ask whether there is α -clustering over and above that given trivially by a particular model of nuclear structure.

The most direct way of studying α -clustering in nuclei is by reactions that knock an α -particle out of the nucleus, or that pull it out in some other way. Among the knockout reactions (p, α) and ($\alpha, 2\alpha$) are the most frequently used, and in addition there are many

transfer reactions such as ($d, ^6\text{Li}$), (^3He , ^7Be) and (^{16}O , ^{20}Ne) that remove an α -particle from the nucleus. Calculations of the cross sections of such reactions can be made using standard nuclear theories, and comparison with the experimental data shows whether there is any excess cross section that could be attributed to α -clustering, since this would automatically increase the likelihood of such reactions.

Some studies of α -clustering have recently been made in this way by Chant, Roos and Wang (*Phys. Rev. C* **17**, 8; 1978) using the ($\alpha, 2\alpha$) reaction at 140 MeV on several light nuclei. They found a large enhancement of the cross sections that strongly indicates the presence of α -clustering.

In the calculation of the ($\alpha, 2\alpha$) cross section, it is necessary to represent the knocked-out α -particle by a wavefunction that is obtained as the eigenstate of a particle in a potential well. The parameters of this potential are chosen to give the known α -particle separation energy and distribution of nucleons in the nucleus, and the quantum numbers of the α -particle state are chosen to conserve the number of harmonic oscillator quanta. A calculation made in this way gave a cross section for the $^{16}\text{O}(\alpha, 2\alpha)^{12}\text{C}(\text{GS})$



A hundred years ago

THE GOVERNMENT of Uruguay intends to construct a railway which will unite Uruguay with the Province of Rio Grande do Sul, in which many thousands of colonists are settled. The line is to begin on the right bank of the Quarahim River, and is to extend as far as the town of Uruguayana. On the Quarahim River this railway will join the line in course of construction between Slatto and Santa Rosa, which is already finished and in use as far as Jacuhi (some 300 miles), and which in turn corresponds with the line between Salto and Fray Bentos, where the great Salderos (slaughter-houses) of the 'Liebig Company' are situated at which over 1,000 head of cattle are killed daily to make the well-known 'Liebig Extract of Meat'.

From *Nature* **18**, 30 May, 133; 1878.

P. E. Hodgson is a lecturer in Nuclear Physics in the University of Oxford.

articles

Long-range transport of tropospheric ozone

Öystein Hov, Eigil Hesstvedt & Ivar S. A. Isaksen

Institute of Geophysics, University of Oslo, Oslo, Norway

Models of ozone and other oxidants are suggested for episodes where air masses of continental origin reach the British Isles during fair weather in summer. It is shown that ozone stays at high levels for several days, allowing for transport over long distances (~1,000 km). Ozone exhibits pronounced diurnal variation in air masses continuously mixed with precursor emissions, while the ozone level is almost constant in ageing air masses due to the rapid drop in NO_x.

CONCENTRATIONS of ozone in the lower troposphere have been observed to increase during periods with anticyclonic weather conditions in the summer season. This occurs over large areas in industrialised countries where the consumption of fossil fuels is substantial and the emissions of hydrocarbons (HC) and nitrogen oxides (NO_x) are high¹⁻⁷. Both observational¹⁻⁷ and theoretical^{8,9} work indicate that urban and rural ozone levels may be strongly coupled. Urban plumes may spread over rural areas while ozone accumulates within the plume. On the other hand, ozone concentrations may increase over periods of several days in rural areas with small emissions of hydrocarbons and nitrogen oxides, given favourable weather conditions.

Tracing ozone-rich air masses back to specific sources is often difficult. In general, the air mass is modified continuously due to mixing with pollutant emissions from ground sources and mixing with other air masses. It is, however, of great interest in oxidant abatement strategies to evaluate to what extent local pollutant levels are caused by nearby or distant sources. Observations by Cox *et al.*^{3,10} focus on measurements at rural sites where influences of local sources can be excluded. Ozone is recorded at Adrigole in Southern Ireland about 300 m from the shore, in episodes where the air comes in over the sea where emissions of hydrocarbons and nitrogen oxides are negligible. The measurements indicate that ozone may be transported over long distances (~1,000 km) and over several days.

We discuss here theoretically the question of long-range transport of ozone. An air mass is exposed to emissions of hydrocarbons and nitrogen oxides for a few days at rates typical of European continental sources. Oxidants would then be expected to develop. Mixing with the surrounding air and deposition on the ground usually takes far longer than the chemical development of the oxidants and can be modelled simply but with sufficient accuracy. The air mass then moves over the sea where the emissions are abolished and ozone deposition is negligible. The predicted ozone levels during the subsequent days are investigated and compared with ozone concentrations measured in similar episodes with transport towards the British Isles.

Model description

The modelled air mass follows the atmospheric mean motion. The vertical extension of the air volume is given by a mixing height H , which exhibits pronounced variations with location and time. At night, there is often a shallow mixing layer next to the ground over land surfaces, and the vertical ozone profile shows significant variation with height, with a strong gradient towards the ground³. The bulk of ozone generated during previous days, however, will remain aloft. We are here concerned with calculations of the bulk concentrations of ozone, and we choose a value $H = 1$ km to represent a characteristic vertical extension over land surfaces of ozone generated from pollutant emissions^{1,3,7,11}. Over the sea the mixing height is computed according to the formula $(4K_z t)^{1/2}$ where K_z is the vertical eddy diffusion coefficient and t is the time elapsed¹². A K_z value of $5 \text{ m}^2 \text{ s}^{-1}$ is chosen.

We assume that all pollutants emitted from ground sources are instantaneously mixed throughout, and that the width of the air mass is large enough to justify that lateral dilution is negligible compared to vertical mixing with cleaner overlying air.

With these assumptions, the continuity equation for any component concentration C may be written as^{8,9,13}

$$(dC/dt) + L_t(C - C_0) = P_c + P_s - L_c C - L_{het} C$$

Here L_t denotes the decay constant due to dilution processes. When the mixing height is assumed constant with time, L_t is equal to the inverse of the characteristic time of turbulent diffusion (τ_o) through the inversion layer at the top of the model volume ($\tau_o = H^2/2K_z'$ where K_z' is the eddy diffusion coefficient in the inversion layer, taken as $0.5 \text{ m}^2 \text{ s}^{-1}$); otherwise $L_t = \Delta H / (H \Delta t)$, where Δt is the time step in the numerical scheme and ΔH the change of mixing height over Δt . C_0 is the concentration in the air surrounding the model volume ('background air', see Table 1). P_c and $L_c C$ are photochemical production and loss terms, respectively. P_s is an emission term defined as Ψ/H , where Ψ is the total flux per unit time of the pollutant in question. The term $L_{het} C$ denotes the loss rate of gas to particle absorptions and ground removal. Ozone and PAN (peroxy acetyl nitrate) are known to be lost through deposition on the ground, in this case $L_{het} = v_d/H$, where v_d is the deposition velocity (about $1-5 \times 10^{-3} \text{ m s}^{-1}$ for ozone, depending on the type of surface, somewhat less for PAN¹⁴). Nitrogen oxides and aldehydes are known to be lost through absorption on particles. In this case L_{het} is taken as constant $3 \times 10^{-6} \text{ s}^{-1}$, corresponding to a characteristic removal time of about 4 d. The predicted oxidant levels are not sensitive to this choice. The continuity

equation is integrated using a version of an explicit numerical scheme previously formulated and validated in detail^{14,15}. This was shown—through comprehensive comparisons with Gear's method^{16,17}—to be an accurate method in the concentration ranges typical of atmospheric pollution and for the type of simulations to be discussed here. It needs only a modest amount of computer time and storage.

The magnitude of anthropogenic emissions of hydrocarbons and nitrogen oxides can be estimated from the consumption of fossil fuels. On average, fluxes of pollutants exceed 1×10^{16} molecules $\text{NO}_x \text{ m}^{-2} \text{ s}^{-1}$ from urban areas in industrialised countries, while they amount to $3\text{--}5 \times 10^{15}$ molecules $\text{NO}_x \text{ m}^{-2} \text{ s}^{-1}$ in countries like West Germany, the Netherlands and UK. The emissions of nitrogen oxides are assumed to consist of 99% as NO and 1% as NO_2 (by volume). The ratio HC/ NO_x is generally >1 (by volume)¹⁸; in urban areas it may amount to

2 or more¹⁸ due to the heavy contribution of car exhaust rich in hydrocarbons. In the model calculations a ratio 1.5 (by volume) is chosen, thought to represent rather well the HC- NO_x emissions from anthropogenic sources in Western Europe¹.

The composition of the hydrocarbon emissions is very complex and varies with type of combustion, or industrial process. A narrow selection must be made as model representation. This is done by choosing a mixture where the wide range of reactivities of hydrocarbons is taken care of by selecting species with very different chemical lifetimes; at one end propene with a lifetime of only a few hours is included, at the other end acetylene and methane are chosen to represent slowly reacting hydrocarbons. The average molecular weight is similar to that commonly found in samples of hydrocarbon pollutants. The formation of oxidants on the time scale typical of long distance transport episodes (several days) does not critically depend on the composition of the hydrocarbon mixture, as long as most of the hydrocarbons are oxidised over this time span. The composition of pollutant emissions of hydrocarbons is given in Table 2.

Fig. 1 *a*, Calculated development of ozone in air masses which follow the atmospheric mean motion and pass over European continental pollution sources of nitrogen oxides and hydrocarbons (the NO_x -flux is 5×10^{15} molecules $\text{m}^{-2} \text{ s}^{-1}$ for curves 1, 4 and 5, a factor 5 down for curve 2, while curve 3 is calculated for background conditions with NO_x -flux 1×10^{14} molecules $\text{m}^{-2} \text{ s}^{-1}$). The mixing height is kept constant as 1 km over the continent, over the sea it increases as $(4K_z t)^{1/2}$. The deposition velocity of ozone over the land is assumed to be $2 \times 10^{-3} \text{ m s}^{-1}$ ($6 \times 10^{-3} \text{ m s}^{-1}$ for curve 4). The calculations are compared with measured diurnal ranges of ozone² in air masses reaching Adrigole, Southern Ireland (A_1 , 14 August; A_2 , 15 August; and A_3 , 16 August 1973). *b*, Calculated development of nitrogen oxides (NO and NO_2), peroxy acetyl nitrate (PAN) and nitric acid (HNO_3) in a long range transport situation (corresponds to curve 1 above).

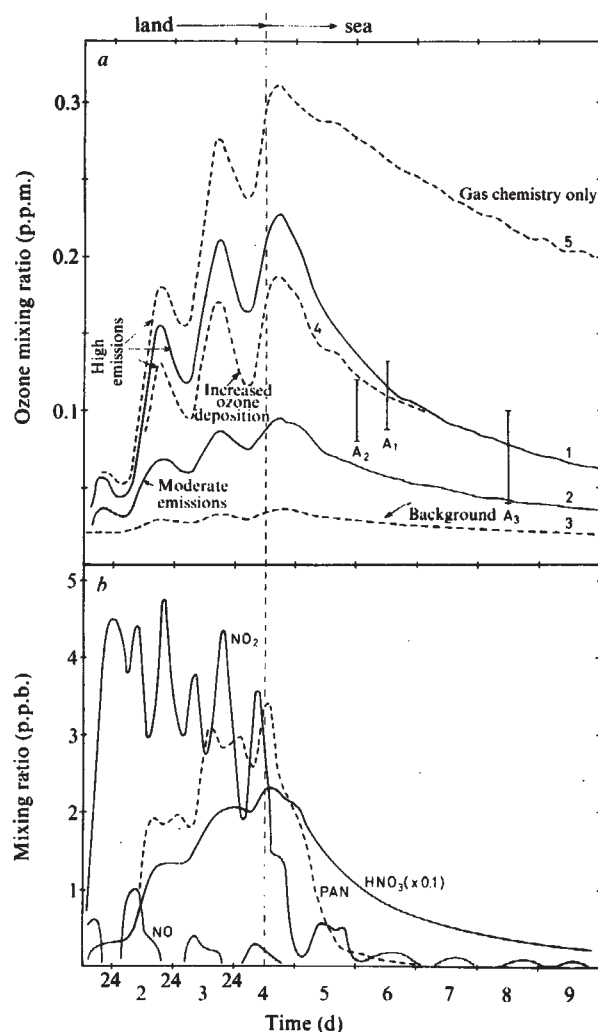


Table 1 Background air mixing ratios

O_3	CO	H_2O_2	NO+ NO_2	HNO_3	CH_4	OH	HO_2
20	300	12	1	5	1,400	8×10^{-5}	5×10^{-3}

Species not mentioned are assumed to have negligible concentrations in background air. Values are in p.p.b. (parts per 10^9).

Table 2 Composition of hydrocarbon emissions (%) of non-oxidised hydrocarbons (by volume)

C_2H_4	C_3H_6	nC_4H_{10}	nC_5H_{12}	C_6H_{14}	C_7H_{16}	CH_4	HCHO	CH_3CHO
20	10	10	10	20	20	10	3	1

Transport of ozone

We follow an air mass which is exposed to emission of hydrocarbons and nitrogen oxides at a rate typical of anthropogenic sources in Western Europe. At noon on the fourth day (72 h of emission) the air mass is transported over sea and the emissions abolished. Over the land, ozone is removed at the ground at a rate of $2 \times 10^{-3} \text{ m s}^{-1}$. This process is negligible over the sea. The development of ozone is shown in Fig. 1*a*. With a NO_x -source strength of 5×10^{15} molecules $\text{m}^{-2} \text{ s}^{-1}$ (corresponding to the average emission in northwestern Europe) we calculate that ozone in excess of 200 p.p.b. is generated, under certain weather conditions (no clouds, midsummer). Furthermore, ozone is kept at a high level for several days. The ozone level is reduced to 100 p.p.b. after about 60 h travel over sea, corresponding to a distance of about 1,100 km at a wind speed of 5 m s^{-1} . The absence of a nocturnal minimum in ozone during the decline reflects the rapid drop in NO_x immediately after the emissions are abolished, as shown in Fig. 1*b*. The NO_x peak on the fifth day amounts to only about 15% of the peak on the fourth day, in strong contrast to the slow decline in ozone. Also, a photochemically active nitrogen compound such as PAN exhibits a rapid drop to less than 10% of its concentration during the accumulation period. Nitric acid is an important end product of gaseous NO_x ; its loss is dominated by heterogeneous processes which act on a longer time scale than the chemical loss of, for example, PAN, hence HNO_3 is kept at elevated levels for days.

Reduced release rates of pollutants lead to lowered ozone maxima. A factor 5 down in the NO_x source strength reduces ozone to about 100 p.p.b. as a maximum still far above the calculated 'background' level of 20–35 p.p.b. (see Fig. 1). The half life, however, is comparable in the two cases. The reduction in ozone is caused by the combined effects of chemical loss and dilution with cleaner air. Curve 5 in Fig. 1 shows the development in ozone when gas kinetic processes alone can occur. By

comparing curves 1 and 5, it is seen that during the accumulation stage ozone is clearly determined by chemistry, whereas during the decline, mixing processes predominate. The effect of an increased ground removal rate of ozone is not significant (curve 4), and an increase in the heterogeneous loss rate of a factor 2 or 3 results in negligible changes in ozone (not shown). The photochemical half life of ozone (as determined from curve 5) is about 1 week.

Observations of ozone at Adrigole in air masses with various transit times over the sea² are shown in Fig. 1. The measured values are seen to be confined rather well within the area bounded by an upper curve corresponding to a high emission rate of NO_x (curve 1) and a lower curve corresponding to a slower release rate (curve 2). The relationship between the release rate of NO_x and the concentrations of ozone must not be taken too literally—an increase of the mixing height by a factor 2 has about the same effect as lowering the release by the same amount, with the assumptions made above.

The slight diurnal variations of ozone observed at Adrigole agree well with the calculations, in contrast to measurements over source areas where the NO_x level is often high enough to induce a marked nocturnal minimum on ozone.

Ozone generated in polluted air masses can be modified by local sources. Some hypothetical situations are shown in Fig. 2a. Emissions are abolished for three days. Then release of HC and NO_x at a rate typical of urban areas¹ takes place for three hours. NO_x levels immediately increase by about 10 p.p.b. Ozone goes up with a few hours time lag; the full effect, however, appears the next day. Due to the elevated NO_x levels, ozone exhibits a marked nocturnal minimum. This agrees with the observed trends⁵.

The efficiency of ozone production depends on the level of pollutants as the urban-type emissions start. About four ozone molecules are produced for every NO_x molecule emitted into a rather polluted air mass (curve 1), in contrast to 10 in the lower case (curve 2) where the level of pollution is modest. In the lower case there is a substantial increase in ozone on the second day after passage over the urban area, reflecting the difference in NO_x (as shown). A pure NO_x source reduces ozone immediately (curve 3). If the air mass can be followed for another day or two, NO_x drops to levels where interaction with methane and other slowly reacting hydrocarbons tend to increase ozone⁹.

A simulation of an episode reported by Cox¹⁰ is shown in Fig. 2b. An air mass passes over central England, with 80 p.p.b. recorded ozone content at Harwell at noon, and reaches Adrigole one day later with an almost constant ozone level of about 80 p.p.b. The calculated ozone pattern agrees well with the observed values. The observed decline in NO_x is not fully reproduced in the calculations; this may be partly due to the simplified emission pattern. Also, the measurements are ground based, whereas we compute bulk concentrations in a volume of 1 km height.

We believe that the model calculations show the principal features of the interaction between photochemistry and dilution in polluted air masses. The photochemical lifetime of ozone in air masses where the precursor emissions are zero is so long (of the order of 10 d) that ozone may be kept elevated for several days, allowing for transport over long distances (around 1,000 km, depending on the wind speed).

Ozone chemistry

The description of the chemical decomposition of the various hydrocarbons in the precursor emissions and the mechanisms responsible for the chemical turnover in the troposphere, are mainly based on data from Demerjian *et al.*¹⁹ and Hampson and Garvin²⁰. We have discussed it in detail elsewhere^{8,9,13,21}.

The rate of ozone formation is closely related to the efficiency of the reactions (R is an organic radical, M is a third body)

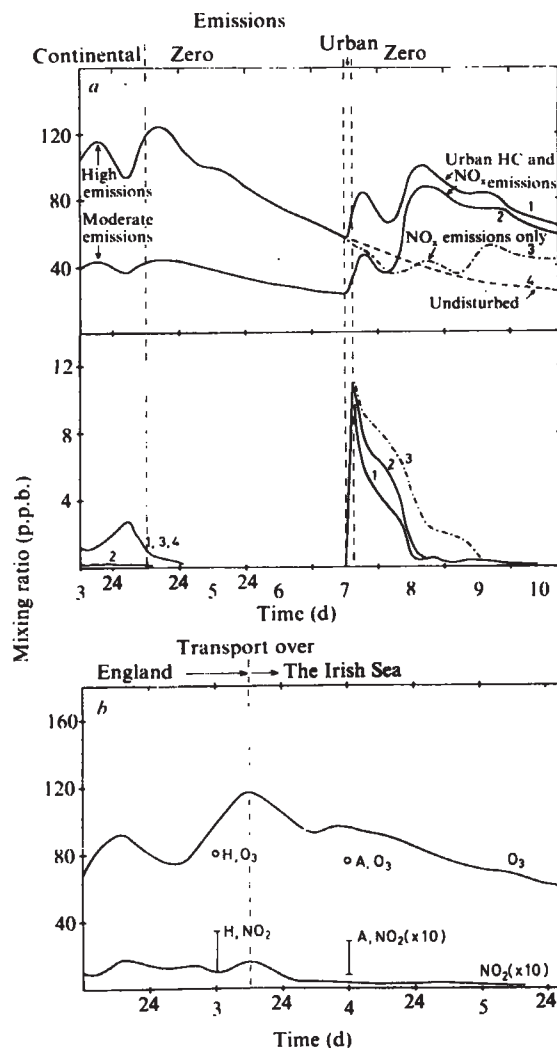
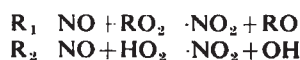
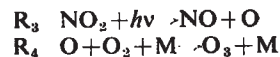


Fig. 2 a, Calculated ozone and nitrogen oxides ($\text{NO} + \text{NO}_2$) in air masses influenced by continental emissions of hydrocarbons and nitrogen oxides (2×10^{15} molecules $\text{m}^{-2} \text{s}^{-1}$ NO_x -flux for curves 1, 3 and 4, a factor 10 down for curve 2) and transported over non-polluted areas for 3 d before they are modified by urban pollutant emissions for 3 h (NO_x -flux 3×10^{16} molecules $\text{m}^{-2} \text{s}^{-1}$ and $\text{HC}/\text{NO}_x = 1.5$; for curve 3 NO_x -emissions only). The mixing height is 1 km throughout, the deposition velocity of ozone is $2 \times 10^{-3} \text{ m s}^{-1}$. b, Simulation of the development of ozone and nitrogen dioxide in air moving over England and passing over Harwell at noon on the third day (pollutant flux of NO_x 2×10^{15} molecules $\text{m}^{-2} \text{s}^{-1}$, 1 km mixing height throughout). The air leaves the west coast of England at 1800 LT, passes over the Irish Sea and reaches Adrigole, Southern Ireland at noon on the fourth day. The calculated values are compared with observations at Harwell (H) 29 August 1974 and Adrigole (A) 30 August 1974¹⁰.

These reactions followed by



form a catalytic set where ozone is generated. Table 3 shows the relative importance of R_1 and R_2 with time. HO_2 interacts closely with other odd hydrogen species (H, OH) and is determined by processes which are active in clean air and intensified in polluted air. Hydroxyl is a key component in air chemistry and is involved in the initial decomposition of most hydrocarbons. Organic peroxy radicals are formed as transitory species during the decomposition of the hydrocarbons. Several HO_2 molecules may be formed when organic peroxy radicals

Table 3 Production and loss processes of ozone, diurnal averages

Process	No. of days	2	3	4	5	6	7	8	9
Production (% of total)									
NO + ΣRO_2 (daytime)		37.0	37.7	37.2	24.7	20.9	15.7	14.6	13.9
NO + HO ₂ (daytime)		63.0	62.3	62.8	75.3	79.1	84.3	85.4	86.1
				$\Psi = 0$					
Loss (% of total)									
NO ₂ + O ₃ (nocturnal loss)		28.7	23.3	9.0	4.0	3.4	3.1	3.2	3.6
NO + O ₃ (nocturnal loss)		7.5	6.0	2.0	0.5	0.4	0.4	0.4	0.5
O ₃ + OH (diurnal loss)		5.4	6.5	7.4	8.6	9.6	9.8	10.1	10.2
O ₃ + HO ₂ (diurnal loss)		21.1	40.0	61.5	70.7	68.6	68.4	66.9	65.2
H ₂ O + O(¹ D) (diurnal loss)		6.4	6.9	8.6	10.6	13.0	13.8	14.7	15.3
NO ₂ + OH (daytime loss)		30.9	17.2	11.5	5.6	4.9	4.5	4.8	5.3
Total loss (molecules cm ⁻³ s ⁻¹)		1.3×10^7	2.3×10^7	2.4×10^7	1.9×10^7	1.5×10^7	1.2×10^7	1.1×10^7	9.8×10^6

$\Psi_{\text{NO}_x} = 5 \times 10^{15}$ molecules m⁻² s⁻¹, HC/NO_x = 1.5 (by volume), gas-kinetic processes only.

are broken down. There is also a recycling of OH to HO₂ through

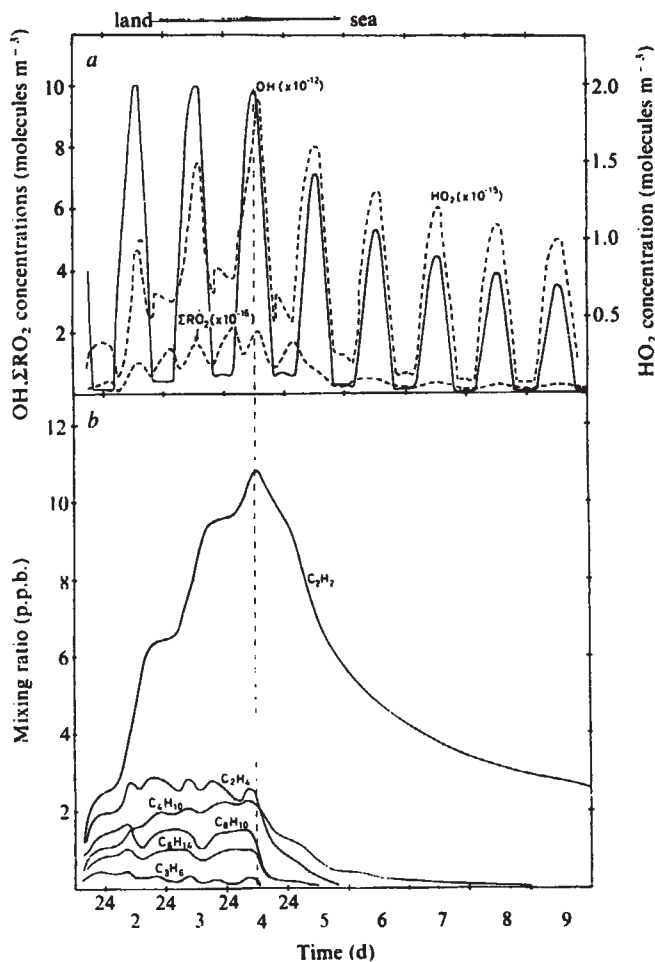


These reactions are effective both in polluted and unpolluted air. In weakly polluted or clean air, water vapour is an important source for hydroxyl radicals through reaction with O(¹D). The level of odd hydrogen is controlled through the loss mechanism



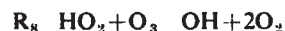
where the feedback to the system from HNO₃ is virtually non-

Fig. 3 Development of radical (a) concentrations and hydrocarbons (b) with time in a long-range transport situation (corresponds to curve 1, Fig. 1a). ΣRO_2 denotes the sum of all organic peroxy radicals.



existent on the time scale of a few days. Figure 3 shows the development with time of the main radicals. The levels of OH and HO₂ do not change significantly during the days which follow a stop in precursor emissions, while organic peroxides decline. The computed levels agree well with observations in polluted air^{22,23}. The reduction in RO₂ reflects the decline of the hydrocarbons (Fig. 3). Figure 3 also demonstrates that the various hydrocarbons act on different time scales with regard to ozone formation. Slowly reacting hydrocarbons such as *n*-butane, acetylene and methane (not shown) contribute larger shares to ozone formation as time elapses, while propene peaks early during the accumulation stage of ozone and drops off quickly as the emissions vanish.

The main loss of ozone during the accumulation stage (Table 3) is caused by a catalytic reaction mechanism active at night when NO_x acts as catalyst¹³, and through the removal of NO₂ during daytime through R₇. As the precursor emissions stop, NO_x drops quickly, and odd hydrogen becomes the main ozone destroying agent, active both day and night. There is a shift in character of the ozone chemistry, resulting in a decline in ozone and absence of a nocturnal minimum. Table 3 shows that reaction



represents approximately two thirds of the total loss of ozone outside polluted areas.

This work is partly sponsored by The Norwegian Research Council for Science and the Humanities (NAVF).

Received 6 February; accepted 13 March 1978.

- Atkins, D. H. F., Cox, R. A. & Eggleton, A. E. *J. Nature* **235**, 372-376 (1972).
- Cox, R. A., Eggleton, A. E. J., Derwent, R. G., Lovelock, J. E. & Pack, D. H. *Nature* **255**, 118-121 (1975).
- Investigation of Rural Oxidant Levels as Related to Urban Hydrocarbon Control Strategies (US Environmental Protection Agency Report EPA-450/3-75-076, 1975).
- 2nd Rep. on the Problem of Photochemical Oxidants and their Precursors in the Atmosphere (OECD, Paris, 1976).
- Apling, A. J. *et al. Nature* **269**, 569-573 (1977).
- Guicherit, R. & van Dop, H. *Atmos. Envir.* **11**, 145-157 (1977).
- White, W. H. *et al. Science* **194**, 187-189 (1976).
- Isaksen, I. S. A., Hesstvedt, E. & Hov, O. *Atmos. Envir.* **12**, 599-605 (1978).
- Isaksen, I. S. A., Hov, O. & Hesstvedt, E. *Envir. Sci. Technol.* (submitted).
- Cox, R. A. *Tellus* **29**, 356-363 (1977).
- Holzworth, G. C. *J. appl. Meteor.* **6**, 1039-1044 (1967).
- Garland, J. A. & Branson, J. R. *Atmos. Envir.* **11**, 659-661 (1977).
- Hov, O., Isaksen, I. S. A. & Hesstvedt, E. *Atmos. Envir.* (submitted).
- Garland, J. A. & Penkett, S. A. *Atmos. Envir.* **10**, 1127-1131 (1976).
- Hesstvedt, E., Hov, O. & Isaksen, I. S. A. *Int. J. chem. Kinet.* (in the press).
- Gear, C. W. *Information processing 68*, 1 (ed. Morrell, A. J. H.) (North-Holland, Amsterdam, 1969).
- Whitten, G. Z. & Meyer, J. P. *Chenik: A Computer Modeling Scheme for Chemical Kinetics* (Systems Applications Inc., San Rafael, California, 1976).
- Roth, P. M., Roberts, P. J. W., Liu, M. K., Reynolds, S. D. & Seinfeld, J. H. *Atmos. Envir.* **8**, 97-130 (1974).
- Demerjian, K. L., Kerr, J. A. & Calvert, J. G. *Adv. Envir. Sci. Technol.* **4** (1974).
- Hampson, R. F., Jr & Garvin, D. *NBS Technical Note 866* (US Dep. of Commerce, Washington, 1975).
- Hesstvedt, E., Hov, O. & Isaksen, I. S. A. *Geophys. Norvegica* **31** (1977).
- Perner, D. *et al. Geophys. Res. Lett.* **3**, 466-468 (1976).
- Einhalt, D. H. *Paper presented at NATO Advanced Study Institute: Arubba, Dolomiti, 13-26 March (1977).*

Role of cell shape in growth control

Judah Folkman & Anne Moscona

Department of Surgery, Children's Hospital Medical Center and The Harvard Medical School, Boston, Massachusetts 02115

Tissue culture plastic adhesivity was precisely varied by applying different concentrations of poly(2-hydroxyethyl methacrylate). The extent of cell spreading was thus accurately controlled so that cells cultured on these substrata could be held at any one of a graded series of quantitated cell shapes. Cell shape was found to be tightly coupled to DNA synthesis and growth in nontransformed cells. These findings suggest a mechanism that is important in growth control of mammalian cells, and provide a more fundamental interpretation of such phenomena as density dependent inhibition of cell growth and anchorage dependence.

OUR hypothesis that appropriate cell shape is critical for DNA synthesis by normal cells¹⁻³ was proposed to explain 'anchorage dependence', a term that describes the inability of normal cells to grow unless attached to a substratum. Most primary fibroblasts proliferate if attached to glass or plastic, but do not grow in suspension culture⁴. Furthermore, we felt that a problem associated with 'anchorage dependence' might also be explained; although normal cells will not grow in suspension culture, they will attach to glass fibrils dispersed in the suspension culture, and proliferation occurs if the fibrils are long enough⁵. However, when the fibrils are less than 20 μm long, proliferation ceases despite the fact that the cells are still anchored. A similar problem is posed by the observation that although many cells do not adhere to bacteriologic plastic, those that do, proliferate very slowly or not at all.

We considered that cell shape was an important variable in all these situations. Cells in suspension culture are spherical; cells on tissue culture plastic are very flat or extremely extended, and cells on bacteriologic plastic, or those attached to glass fibrils less than 20 μm long, maintain a foreshortened configuration that lies somewhere between spherical and flat. Despite its explanatory value, there was little quantitative data to support this theory. There was no easy way to hold cells indefinitely, at various degrees of spreading. Also, comparative measurements of cell shape were cumbersome. We have now solved these difficulties, and describe here new experimental evidence in support of this hypothesis.

Control of cell shape by variation of substratum adhesiveness

We have found that cell shape can be precisely varied by a simple modification of substratum adhesiveness. The adhesiveness of plastic tissue culture dishes can be permanently reduced in a graded manner by applying increasing concentrations of poly(2-hydroxyethyl methacrylate) (poly(HEMA)). When an alcoholic solution of poly(HEMA) is pipetted into a plastic culture dish, a thin, hard, sterile film of optically clear polymer remains tightly bonded to the plastic surface after the alcohol evaporates. Serial dilutions of the alcohol polymer solution, introduced into each dish at a constant volume, result in decreasing thicknesses of the polymer film. These films vary between 35 and 0.0035 μm in thickness, with a correspondingly increased adhesivity. Cells plated on the thickest layer of

polymer (35 μm) are the least adherent, and most closely approach a spheroidal configuration. On thinner polymer layers, the cells become flatter (Fig. 1). At least 12 cell conformations from spherical to flat can be achieved by this method. Once a population of cells has reached shape equilibrium, the cells maintain an average shape, specific for each polymer thickness, throughout an experiment and for at least 7 d.

Cell shape was measured by two methods: a horizontal dimension (cell diameter) was measured with the micrometer

Dilution of poly (HEMA) solution	Mean cell diameter (μm)	Dilution of poly (HEMA) solution	Mean cell diameter (μm)
1.00	10 $\pm$ 2	6×10^{-3}	27 $\pm$ 8
1×10^{-1}	9 $\pm$ 2	4×10^{-3}	33 $\pm$ 10
8×10^{-2}	9 $\pm$ 2	1×10^{-3}	37 $\pm$ 12
4×10^{-2}	16 $\pm$ 6	5×10^{-4}	46 $\pm$ 11
1×10^{-2}	24 $\pm$ 7	1×10^{-4}	46 $\pm$ 13
8×10^{-3}	27 $\pm$ 11	Plastic	51 $\pm$ 7

Fig. 1 Variation of cell diameter with thickness of poly(HEMA) substratum. The 2.1-cm² wells of Falcon 3008 culture plates were coated with dilutions of poly(HEMA). 6 g poly(HEMA) was dissolved in 50 ml 95% ethanol and the mixture turned slowly overnight at 37 °C to dissolve the polymer completely. The viscous solution was centrifuged for 30 min at 2,500 r.p.m. to remove undissolved particles. This stock was then diluted with 95% ethanol from 10⁻¹ (that is, 1.00 ml poly(HEMA) + 9.00 ml ethanol) to 10⁻⁴. 200 μl was pipetted into the microwells so that each concentration was represented in quadruplicate. Sterile technique was used in a 37 °C warm room and plates were dried on a level bench free of vibrations. The wells were allowed to dry for 48 h with the lids in place. If there was a break in sterility, exposure to ultraviolet light before use did not harm the poly(HEMA) films. Bovine aortic endothelial cells²¹ were plated at 15,000 per cm², and incubated for 24 h. 20 cells in each well were then measured with a Nikon profile projector at $\times 200$. The longest axis of each cell body was measured; pseudopod extensions were not included in the measurement.

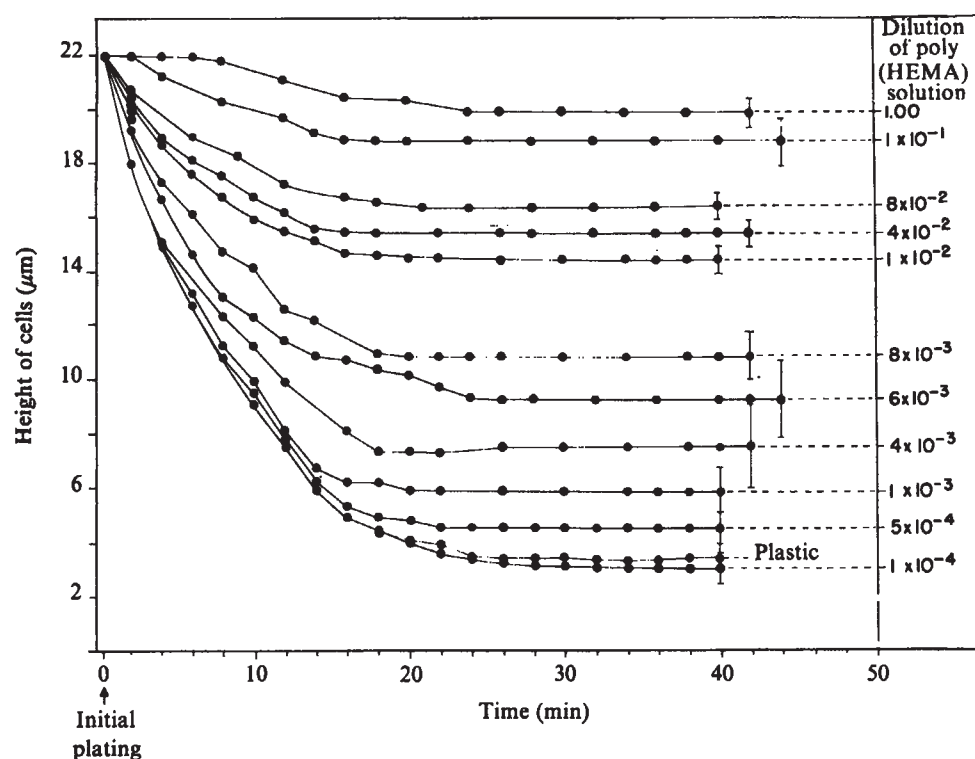


Fig. 2 Variation of cell height with thickness of poly(HEMA) substratum immediately after cells are plated. The micrometer of an inverted phase microscope was connected by a pulley to an angular transducer which was fed to a digital read-out device calibrated to read directly in μm . Poly(HEMA) films of graded thickness were prepared as in Fig. 1. Endothelial cells were plated at 10,000 per cm^2 per well. Just before adding the cells, a fine scratch was made on the poly(HEMA) surface. The lid was sealed with parafilm and the plate was placed on the microscope stage at 37°C . The height of each of 5 cells was measured to $\pm 0.1 \mu\text{m}$ by focusing on the body of the cell. Each experiment was repeated in quadruplicate. The shape at equilibrium did not change over a 7-d period. Although poly(HEMA) films swell slightly after hydration, all thicknesses of poly(HEMA) reached swelling equilibrium less than 1 min after media were added.

stage of a Nikon profile projector (Fig. 1), and a vertical dimension (cell height) was obtained by using the fact that cells freshly plated on plastic continually go out of focus as they flatten and spread. Therefore, the fine-focus micrometer on a standard inverted phase microscope was attached to a rotary transducer so that measurements in the vertical dimension could be recorded by digital read-out to $\pm 0.1 \mu\text{m}$. This permitted rapid measurement of the height of a cell, its height decreasing as the cell flattened. The floor of the culture dish was calibrated to 'zero' by making fine scratches on it with a glass knife or a glass wool swab, and also by focusing on microspheres of $20 \mu\text{m}$ diameter resting on the surface. The time required for cells to reach shape equilibrium by measuring the vertical dimension is shown in Fig. 2.

Variation of DNA synthesis with cell shape

The number of cells capable of DNA synthesis was determined for the population of cells on each thickness of poly(HEMA) substratum by incubating the cells with ^3H -thymidine after they had reached shape equilibrium. For all three lines tested (bovine endothelial cells, WI-38 cells and A-31 cells), incorporation of ^3H -thymidine was found to be inversely proportional to the height of the cell (Fig. 3). Autoradiographs were also made of each cell population and confirmed the results from scintillation counting (Figs 4 and 5). These experiments, in which cells were not crowded and in fact were not in contact with each other, led to the following question. If DNA synthesis is inhibited as cells change toward a more spheroidal conformation, could this shape modulation account for the

decrease in DNA synthesis associated with crowding of cells? It is well known that DNA synthesis gradually stops as cells become confluent⁶. It has also been shown⁷ that mitoses continue for a little while after contact. It was largely for this reason that Stoker and Rubin proposed the term 'density dependent inhibition of growth'⁸.

Is density dependent inhibition of cell growth mediated by cell shape?

Endothelial cells were plated at various densities on tissue culture plastic. As the cells grew to confluence, they came out of focus in the reverse order from their plating as each cell was laterally compressed by its neighbours. However, this change occurred over several days. The most confluent cells attained the greatest mean height and also incorporated the least amount of ^3H -thymidine. The most striking finding, however, was that whenever a poly(HEMA) substratum was chosen with the appropriate adhesivity to hold sparsely plated WI-38 cells at the same height as their confluent counterparts on plastic, the incorporation of ^3H -thymidine for the two populations was similar (Table 1). In other words, sparse cells held at rounded shapes approached levels of DNA synthesis that closely matched that of confluent cells. In this experiment, cell shape was driven toward the spheroidal configuration by two different mechanisms: first, the cells on poly(HEMA) were not in contact with each other but they became rounded or hexagonal because of a reduction in substratum adhesiveness; second, by contrast, the cells on plastic were on a maximally adhesive substratum

Table 1 Effect of crowding on cell shape and DNA synthesis (WI-38 cells)

Poly (HEMA)			Plastic		
Dilution of poly (HEMA)	Cell density (cells per cm^2)	^3H Incorporation (c.p.m.)	Cell height (μm)	Cell density (cells per cm^2)	^3H Incorporation (c.p.m.)
1.00	15,000	7 ± 5	22	250,000 (confluent)	7 ± 1
4×10^{-2}	15,000	50 ± 5	15	100,000 (confluent)	55 ± 9
4×10^{-3}	15,000	250 ± 15	6	30,000 (subconfluent)	254 ± 23

WI-38 cells were plated at the densities indicated on uncoated plastic (Falcon 3008) and also on a series of poly(HEMA) substrates of graded thickness. Cell height, number and ^3H -thymidine incorporation (c.p.m.) were measured as in Fig. 3. When poly(HEMA) plates of different adhesivity containing sparsely plated cells were selected for cells whose height most closely matched the height of cells on plastic at various densities, the incorporation of ^3H -thymidine was more equivalent for these pairs of plates than for any other combination of plates. Similar results were obtained with endothelial cells.

but could not flatten because they were being crowded by their neighbours. However, the resulting reduction in DNA synthesis was similar in both situations. This experiment was repeated with endothelial cells with similar findings.

The result was substantiated by a second experiment. Swiss 3T3 cells were sparsely plated on bacteriologic plastic, and their mean height and percentage ^3H -thymidine incorporation was measured. Swiss 3T3 cells were also seeded on tissue culture plastic at pre-confluent density and allowed to grow to a density at which their average height was equivalent to the height of the cells on bacteriologic plastic, then ^3H -thymidine incorporation was determined. It was evident that the crowded cell population on tissue culture plastic with a mean height most closely approximating to the height of sparse cells on bacteriologic plastic, contained a ratio of DNA synthesising cells that most closely matched the population on bacteriologic plastic (Table 2).

The idea that density dependent inhibition of growth is partly mediated by cell shape was then corroborated by a third experiment. When wounds are made in a confluent monolayer of cultured cells, the surviving cells at the wound edge, and those in a few rows behind the edge, show an increased incorporation of ^3H -thymidine. After the cells have closed the gap, DNA synthesis returns to the low level of the other confluent cells. Therefore, wounds were made in a confluent monolayer of A-31 cells cultured on plastic. Focal depth measurements that began immediately after wounding showed that the first row of cells at the wound edge began to flatten within 30-60 min, as these cells gradually spread out onto the exposed substratum (Fig. 6). Within one hour the first row of cells had flattened by

Fig. 3 Variation of DNA synthesis with cell shape: Endothelial cells. Endothelial cells were plated on poly(HEMA) substrates and plastic as in Fig. 2. Four wells were used for each poly(HEMA) dilution. Cell height was measured in the 1st well. Cells in the 2nd well were counted at the end of the experiment (48 h). Cells in the 3rd and 4th wells were exposed to ^3H -thymidine ($1 \mu\text{Ci ml}^{-1}$) for 42 h, and c.p.m. per 1,000 cells were determined on precipitated DNA. Similar results were obtained with WI-38 and A-31 cells. The wide standard deviations in wells of poly(HEMA) 10^{-2} to 10^{-3} are partly due to microscopic ripples at the periphery of each well, mostly related to rapid drying of the polymer film.

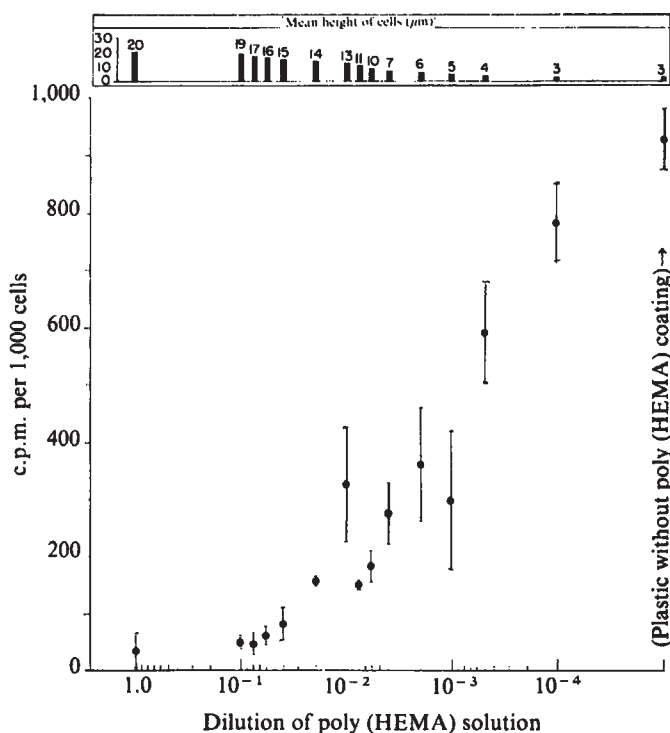


Table 2 The effect of crowding on cell shape and DNA synthesis (Swiss 3T3 cells on bacteriologic plastic compared with tissue culture plastic)

Time after plating (h)	No. of cells	c.p.m. per 1,000 cells	Mean height (μm)	Range
Bacteriological dishes				
60	1.26×10^5	76.9	12	(8-14)
72	1.51×10^5	60.0	12	(8-14)
94	1.93×10^5	45.0	12	(8-14)
Tissue culture dishes				
60	2.63×10^5	132.0	5	(1-8)
72	3.00×10^5	51.2	12	(9-14)
94	3.85×10^5	17.8	17	(10-20)

27 bacteriological dishes (Falcon 1008) and 27 tissue culture dishes (Falcon 3005) were seeded with 5×10^4 cells. Three dishes in each group were scratched for cell height measurements. At each time point, the mean cell height is the average for 20 cells, with 10 measurements per cell. Cell number and ^3H -thymidine incorporation were also measured at each time point. ^3H -thymidine incorporation in Swiss 3T3 cells grown to confluence on tissue culture plastic until they were sufficiently crowded to resemble the shape of sparsely plated cells on bacteriologic plastic, was equivalent to the ^3H -thymidine incorporation of sparse cells on bacteriologic plastic.

54% of their original height, the next row by 42% and the third row by 30%. Beyond that, cells were still at the same height as the remainder of the confluent layer. Although ^3H -thymidine was not added in this experiment, the expected labelling index for A-31 cells of these respective shapes would predict the highest labelling index for the first row with a subsequent decrease in labelling of the cells further back.

Controls

It might be argued that the decreasing DNA synthesis in cells plated on thicker poly(HEMA) layers could be an artefact of sublethal cytotoxicity on the part of the polymer. This possibility was ruled out by five experiments: (1) glass coverslips coated with poly(HEMA) were placed on top of cells that had already been plated on plastic. Time lapse films showed that cells directly beneath the poly(HEMA) had the same mitotic rate as cells remote from it. (2) Glass cylinders were coated with thick poly(HEMA) and cemented to plastic dishes which were then seeded with cells. Autoradiographs showed that cells growing on the plastic surface came directly in apposition to the wall of poly(HEMA) without any zone of decreased ^3H -thymidine labelling. (3) Deep scratches were made in thick poly(HEMA) layers. The cells would not spread or grow on top of the poly(HEMA), but they did stretch and proliferate deep in the scratched troughs, while surrounded by poly(HEMA). (4) Endothelial cells were plated in wells in which the substratum was either thick poly(HEMA) ($35 \mu\text{m}$) or 1.0% Agarose. Cells settled on the surface but could not attach, and continued to roll around. Every day cells were 'rescued' from a poly(HEMA) well and from an Agarose well by aspiration with a pipette, and plated on tissue culture plastic. For each day that cells had remained spherical, plating efficiency on plastic decreased. By the 8-9th day, plating efficiency was zero, and cells from both the poly(HEMA) wells and the Agarose wells were considered dead. This indicated that poly(HEMA) per se is as nontoxic as Agarose, and that the eventual loss of plating efficiency was the result expected for nontransformed cells maintained in the completely rounded state (like suspension culture). (5) Finally, transformed cells (SV 3T3) grew almost as rapidly on thickest poly(HEMA) as on 10^{-4} polymer or plastic despite the fact that their cell shape varied with substrate adhesiveness in the same proportion as for nontransformed cells. This indicates that in transformed cells, cell shape and cell growth may be uncoupled.

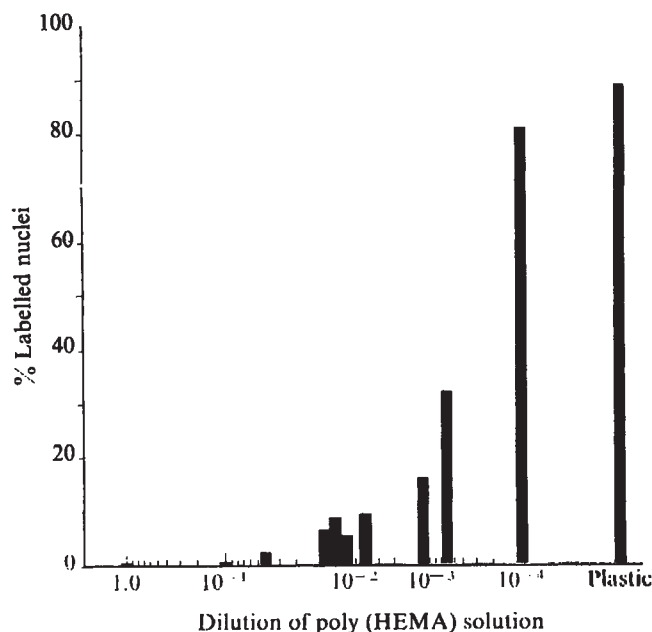


Fig. 4 Variation of DNA synthesis with cell shape: Endothelial cells (autoradiography). Cells were treated as in Fig. 3, except that at the end of ^3H -thymidine incubation, all the cells in each well were removed by brief trypsinisation to a corresponding microwell (Falcon 3040) of uncoated plastic. After 2 h, these attached cells were prepared for autoradiography, developed after 5 d, and 400 cells in each well counted, and expressed as %, labelled nuclei. Similar experiments were carried out with WI-38 and A-31 cells.

Discussion

Our experiments show that for nontransformed mammalian cells, the shape of the cell is tightly coupled to DNA synthesis. As cells of various lines are brought from the extremely flat shape toward a spheroidal conformation, fewer cells incorporate ^3H -thymidine. Finally, when cells are almost completely spherical, but still barely attached to the substratum, they fail to enter the S phase, analogous to floating cells. Cell shape is controlled either by substratum adhesiveness or by the combination of substratum adhesiveness and crowding by neighbouring cells. It should be emphasised that time-lapse films show that for substrata of low adhesivity, equilibrium shape is actually the average shape maintained by each cell as it continually spreads out and retracts. However, even the extent of this transient spreading is less when the adhesivity of the substratum is diminished.

The mechanism by which poly(HEMA) reduces the adhesivity of polystyrene is not entirely known. Poly(HEMA) is a hydrophilic hydrogel of neutral charge. It may act by reducing the net negative electrostatic charge of the polystyrene, but other mechanisms are tenable. Although scanning electron micrographs of all thicknesses of poly(HEMA) showed a smooth homogeneous surface, it is possible that tiny spicules of plastic, below the resolution of the scan, protrude as multiple contact points to which cells stick. Increasing thicknesses of poly(HEMA) would then act to decrease the number of available contacts with plastic. The important conclusion is that whatever the mechanism by which poly(HEMA) can reduce adhesivity of polystyrene, cells respond by a change in shape.

These experiments suggest that several different phenomena of cell culture may be mediated by cell shape. For cells subject to anchorage dependence, shape is modulated by substrate adhesiveness, and only when cells are spread to the appropriate degree can DNA synthesis proceed. Density dependent inhibition of growth may result from the close packing of cells by their neighbours, result-

ing in a cell shape that does not allow DNA synthesis. As cells become confluent, mitoses may continue briefly after cell contact because of the time required to bring about close cell packing. The subsequent cell shape changes apparently occur as the pressure generated by each mitosis is attenuated throughout the monolayer. Zetterberg and Auer described the reduction in area occupied by each cell as cell density increased⁸. Furthermore, the stimulation of cell growth by wounding of a monolayer may also result from modulation of cell shape at the wound edge. As the leading edge of cells spreads out onto the open substratum, cells in the interior seem to become unpacked, and partially begin to stretch and flatten although to a much lesser degree than cells at the edge (Fig. 6). Dulbecco's finding¹⁰ that cells at the wound edge have a higher labelling index than cells in the next interior row may be the result of this stepwise unpacking and flattening. Additional evidence is found in the demonstration that the spreading and movement of a whole row of cells into an open wound precedes DNA synthesis¹¹.

The relation of serum growth factors to cell shape

If cell shape is so tightly associated with the onset of DNA synthesis, how then is the influence of serum growth factors to be explained? The addition of fresh serum or of purified growth factors¹² or the increased velocity of medium flow¹³ can sometimes overcome the restriction of growth imposed by high cell density. This has led to a humoral hypothesis which suggests that high cell density limits the availability of medium components such as growth factors present in the serum¹⁴. However, the humoral hypothesis cannot explain the inhibition of cell growth in sparse cultures on substrata of low adhesivity (that is, poly(HEMA) 10^{-2}).

This difficulty can be resolved by considering that growth control associated with cell shape and the sensitivity of cells to growth factors may be linked. Thus, higher concentrations of growth factors would be required for DNA synthesis as cell conformation was converted from flat toward hexagonal, and finally to spheroidal, where no concentration of serum would be sufficient for growth. There is

Fig. 5 Variation of DNA synthesis with cell shape: A-31 cells (autoradiography). BALB/3T3 (A-31) cells (late passage) were incubated with ^3H -thymidine as in Fig. 4. In contrast to endothelial cells, this A-31 population already has a few cells able to grow without anchorage. Further passage from high density might be expected to select transformed cells capable of growth in suspension culture.

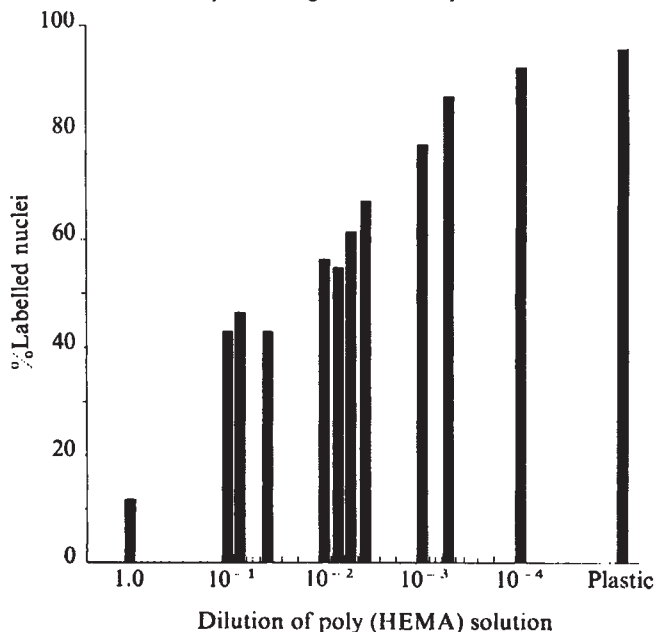




Fig. 6 Change in cell shape after wounding of confluent monolayer. BALB/3T3 (A-31) cells (early passage) were allowed to grow to early confluence on tissue culture plastic (Falcon 3001), on which a fine scratch had previously been made for cell height calibration. The height of cells over the whole plate before wounding was $14.8 \pm 0.5 \mu\text{m}$. One hour after the monolayer had been wounded with a glass knife, cell heights were measured again. Cells at the wound edge had spread out and flattened down to $6.8 \mu\text{m}$. Cells in the next interior row had also begun to flatten, but to a lesser extent and without loss of contact.

scattered evidence for this idea in the literature. Such a relationship between cell shape and sensitivity to growth factors would explain why cells at the edge of a wound require less serum for DNA synthesis than cells a few rows behind them¹⁰. Also, this concept would explain why BHK21 cells grown on plastic were 60 times more sensitive to serum than the same cells grown in suspension culture¹³ and why Paul *et al.* found serum requirements to be reduced in anchored cells¹⁶. This hypothesis could be tested by culturing cells on substrata of graded adhesivity with increasing serum concentrations.

Theoretical considerations

These data do not distinguish whether cell shape 'controls' cell proliferation or whether both parameters are dependent on a third factor related in some way to the substrate. Also unclear are the important variables associated with the rounded cell that prevent proliferation. The disappearance of bundles of microfilaments¹⁷, the decreased attachment points to the substratum, or the reduction in the rate of transport of certain nutrients^{18,19} are all possibilities. The method of holding large populations of cells at any given average shape, as described here, may provide a new approach to these problems. Because transformed cells can proliferate without anchorage²⁰ or while being held in any cell shape, this difference from nontransformed cells may also be amenable to study by poly(HEMA). It is possible that the various stages of transformation will be classifiable in a more quantitative way according to the ability of cells to grow on increasing thicknesses of poly(HEMA).

Finally, normal cell growth *in vivo* may possibly be regulated by a complex balance of adhesive forces between cells and between cell and substratum (that is, basement membrane) that combine to modulate cell shape, and permit or prevent DNA synthesis. In contrast, a hallmark of malignancy is continual proliferation despite cell crowding. We have reported the importance of angiogenesis capacity for tumour cells to surmount the diffusion problems of crowding²². The present study suggests an antecedent property; the capacity of a tumour cell to overcome the effects of close cell packing and thus continue DNA synthesis.

We thank Drs A. Kornberg and R. Tucker for discussions, Drs A. Pardee, R. Pollack and H. Green for reviewing this manuscript, Drs D. Ausprunk and C. Handenschild for the scanning electron micrographs, J. Wilson, Mrs C. Butterfield, B. Smith and C. Cobb for assistance, Mrs P. Breen for typing the manuscript and Dr S. Ronel (Hydron) for poly(2-hydroxyethyl methacrylate). This work was supported by grant CA14019-04 from the US NCI and a grant from Monsanto Co.

Received 19 December 1977; accepted 14 April 1978.

1. Folkman, J. & Greenspan, H. P. *Biochim. biophys. Acta* **417**, 211-236 (1975).
2. Folkman, J. in *Advances in Pathobiology, Cancer Biology II, Etiology and Therapy* Vol. 4, (eds Fenoglio, C. & King, D. W.) 12 (Stratten Intercontinental, 1976).
3. Folkman, J. in *Recent Advances in Cancer Research: Cell Biology, Molecular Biology, and Tumour Virology* Vol. 1 (ed. Gallo, R. C.) 119-130 (CRC, Cleveland, 1977).
4. Stoker, M. G. P., O'Neill, C., Berryman, S. & Waxman, V. *Int. J. Cancer* **3**, 683-693 (1968).
5. Maroudas, N. G., O'Neill, C. H. & Stanton, M. F. *Lancet* **i**, 807-809 (1973).
6. Zetterberg, A. & Auer, G. *Expl Cell Res.* **62**, 262-270 (1970).
7. Martz, E. & Steinberg, M. S. *J. Cell Physiol.* **79**, 189-210 (1972).
8. Stoker, M. G. P. & Rubin, H. *Nature* **215**, 171-172 (1967).
9. Dulbecco, R. & Stoker, M. G. P. *Proc. natn. Acad. Sci. U.S.A.* **66**, 204-210 (1970).
10. Dulbecco, R. *Nature* **227**, 802-806 (1970).
11. Sholley, M. M., Gimbrone, M. & Cotran, R. *Lab. Invest.* **36**, 18-25 (1977).
12. Mierzejewski, K. & Rozengurt, E. *Nature* **269**, 155-156 (1977).
13. Stoker, M. G. P. *Nature* **246**, 200-203 (1973).
14. Holley, R. W. *Nature* **258**, 487-490 (1975).
15. Clarke, G. D., Stoker, M. G. P., Ludlow, A. & Thornion, M. *Nature* **227**, 798-801 (1970).
16. Paul, D., Henahan, M. & Walter, S. *J. natn. Cancer Inst.* **53**, 1499-1503 (1974).
17. Bragina, E. E., Vasiliev, Ju. M. & Gelfand, I. M. *Expl Cell Res.* **97**, 241-248 (1976).
18. Pardee, A. B. *Natn. Cancer Inst. Monogr.* **14**, 7-20 (1964).
19. Bissell, M. J., Farson, D. & Tung, A. S. C. *J. Supramolec. Struct.* **6**, 1-12 (1977).
20. Shin, S., Freedman, V., Risser, R. & Pollack, R. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4435-4439 (1975).
21. Birdwell, C. R., Gospodarowicz, D. & Nicolson, G. L. *Nature* **268**, 528-531 (1977).
22. Folkman, J. & Cotran, R. in *Int. Rev. exp. Path.* **16** (eds Richter, G. W. & Epstein, M. A.) 207-248 (Academic, New York, 1976).

Isolation by molecular cloning of a fragment of the split ovalbumin gene

A. C. Garapin*, J. P. Lepenne†, W. Roskam*, F. Perrin†, B. Cami*, A. Krust†, R. Breathnach†, P. Chambon†, & P. Kourilsky*‡

* Groupe de Biologie Moléculaire du Gène, ERA CNRS 201 Institut Pasteur, Paris, France

† Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS et U44 de l'INSERM, Faculté de Médecine, Strasbourg, France

An EcoRI fragment of chicken DNA (fragment 'a') containing a sequence complementary to the 3' half of ovalbumin mRNA has been isolated by molecular cloning. Analysis of the cloned fragment proves conclusively that the chicken ovalbumin gene is split. Fragment 'a' contains no extensive sequence repeated elsewhere in the genome and represents the only type of organisation of this part of the split ovalbumin gene in chicken genome.

‡ To whom correspondence should be addressed.

THE discovery of mosaic adenovirus and SV40 mRNAs (see refs 1 and 2 and references therein) has raised the question of whether cellular mRNAs could also be coded by separate blocks of DNA sequences. In a recent report³ we have shown that coding sequences of the ovalbumin gene are interrupted at least twice in the chicken genome; similar results were presented by Doel *et al.*⁴. It was reported almost simultaneously that several eukaryotic genes consist of blocks of coding sequences separated by intervening sequences⁵. Thus, an intervening sequence has been found within the coding region of the rabbit and mouse β globin genes⁶⁻⁸, and the variable and

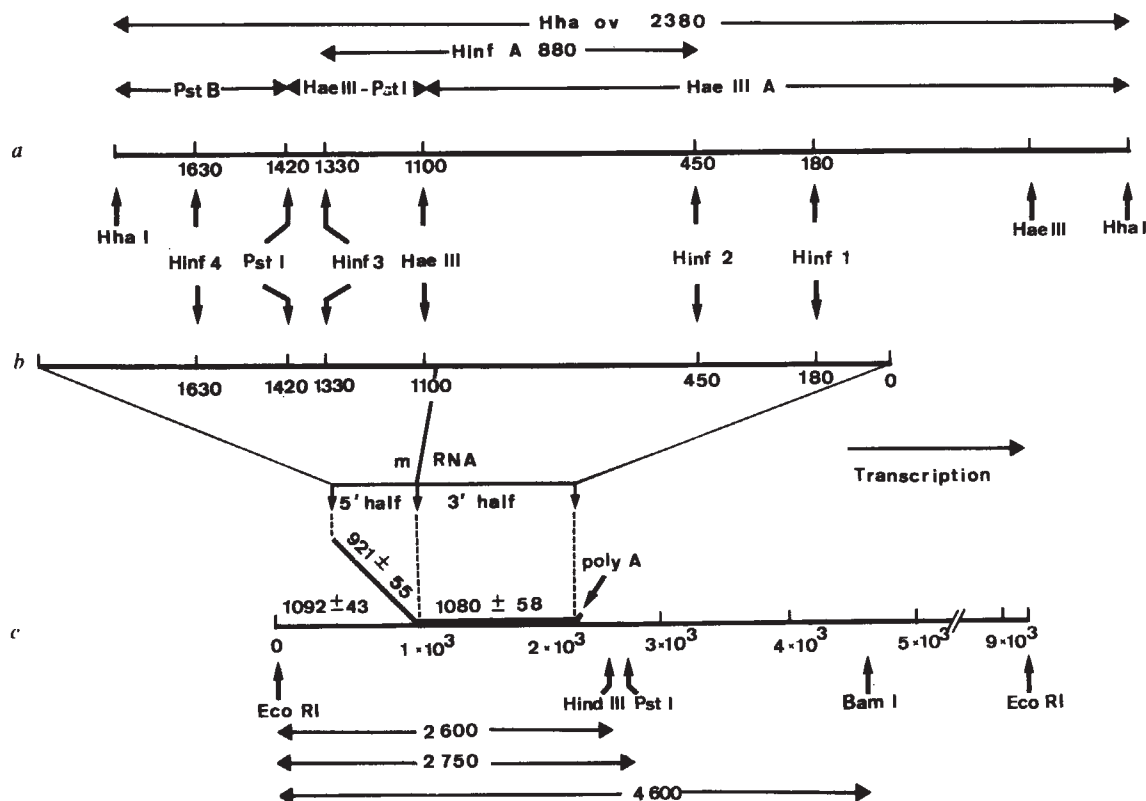


Fig. 1 *a*, Physical map of restriction enzyme sites in the *Hhaov* fragment³. The positions of the restriction endonuclease sites in the *Hhaov* fragment are given from the 3' end of the mRNA (excluding the poly A tail). The localisation and the sizes of the fragments which have been used as hybridisation probes are also shown. *b*, Physical map of the relevant restriction endonuclease sites in the *in vitro* synthesised ovalbumin ds-cDNA (see ref. 3). The position of the restriction sites are given from the 3' end of the ov mRNA (position 0). The lines and the arrows which are below the map indicate which part of the ds-cDNA is contained in fragment a, where it codes for the 3' half of ov mRNA, and is contained in other regions of the split ovalbumin gene where it codes for the rest of ov mRNA. *c*, Schematic representation of the RNA-DNA hybrid molecules shown in Fig. 6a and b. For the positions of the restriction enzyme sites and length determinations by agarose gel electrophoresis and electron microscopy, see text and Figs 4 and 6.

constant gene sequences within the coding region of a cloned mouse plastocytoma λ light chain are separated by an intervening sequence of 1,250 base pairs⁹. Intervening DNA sequences are not confined to mRNA coding genes, as in addition to the *Drosophila* ribosomal DNA insertions^{2,10-13}, Goodman *et al.*¹⁴ and Valenzuela *et al.*¹⁵ have reported that yeast tRNA genes contain short intervening sequences. It seems, therefore, that the split gene organisation may be a general feature in eukaryotic cells.

To further understand the structural and functional organisation of split genes, we set out to isolate the fragments of the ovalbumin gene by molecular cloning. Phage systems for the cloning of *Eco*RI DNA fragments present many advantages, so we chose as our starting material the *Eco*RI fragment 'a' of chicken cellular DNA, which is about 9,000 base pairs long and contains the DNA sequence coding for the 3' half of the ovalbumin mRNA (ov mRNA) (ref. 3). As a first step towards the isolation of the entire ovalbumin gene, we report here the cloning, isolation and characterisation of fragment a, providing direct evidence for the split organisation of the ovalbumin gene.

Cloning of the ovalbumin gene fragment 'a' from chicken DNA

In a previous study¹⁶, the purified ov mRNA (about 1,900 nucleotides), was transcribed into complementary double stranded DNA (ds-cDNA), integrated into a bacterial plasmid and cloned in *Escherichia coli*. One plasmid, pCRIov2.1, carries

a 1,730-base pair sequence coding for ov mRNA. Digestion of the plasmid DNA with *Hha*I endonuclease yields a 2,380-base pair fragment (*Hhaov*, see Fig. 1), which contains the cloned ovalbumin gene sequence. The *Hhaov* fragment was used to prepare highly radioactive probes.

*Eco*RI cleaves chicken DNA in 300,000 to 400,000 fragments, most of which are smaller than molecular weight 2×10^6 (W.R., in preparation). Fragment a is much larger, so we used preparative gel electrophoresis with continuous elution (W.R., in preparation) to obtain fractions enriched in fragment a. As shown in Fig. 2, filter hybridisation of the various fractions with the *Hhaov* probe revealed a peak corresponding to DNA with the expected length of about 9,000 base pairs. The size distribution of DNA in the peak fractions was analysed by electron microscopy and compared with that of unfractionated DNA. From these data, it was estimated that fragment a present in the peak was enriched at least 20-fold with respect to mass or 50-fold with respect to the number of fragments. 0.5 μ g of this enriched DNA was recombined *in vitro* with the purified arms of the λ gtWES. λ C vector¹⁸ from which the central λ C fragment had been removed. After *in vitro* packaging¹⁹, 800,000 independent recombinant phages were produced (each recombinant originates from a different *in vitro* recombination event). A total of 30,000 phages were screened on 10 plates by the *in situ* hybridisation procedure described in the legend to Fig. 3. Ten spots were observed on the autoradiograms (Fig. 3a). The phages in the corresponding areas were picked and re-isolated. A second *in situ* hybridisation was carried out on plates carrying not more than a few hundred phage plaques. Three independently isolated phages yielded positive plaques in

this second *in situ* hybridisation (Fig. 3b). The three corresponding phages, B, C and D, were grown on plates, then in liquid, and purified by CsCl centrifugation. As expected, the purified phage DNA hybridised readily with labelled *Hhaov* probe and ovalbumin cDNA (not shown).

The cloned DNA is the *EcoRI* ovalbumin gene fragment a

Several lines of evidence indicate that all three independent clones B, C and D harbour the ovalbumin gene fragment a.

Agarose gel electrophoresis of *EcoRI* digests of all three B, C and D DNA indicated the presence of a fragment of about 9,000 base pairs in addition to the two λ arms (not shown). The length of the integrated fragment was confirmed by electron microscopic measurements on the three *EcoRI* fragments of clones B and C, using Φ X174 as a standard. A value of $9,230 \pm 350$ base pairs was found for the integrated fragment, whereas values of $21,400 \pm 400$ and $14,600 \pm 400$ base pairs were found for the left and the right arms of λ vectors, respectively, in good agreement with previous estimation^{18,26}. As expected, hybridisation with the *Hhaov* probe was restricted to the 9,200-base pair fragment. This was demonstrated by an experiment in which the *EcoRI* digest was transferred from the gel to a nitrocellulose filter²⁷ before hybridisation and autoradiography³ (Fig. 4, lanes 2–4).

Using the blotting technique outlined above, the 9,200 base pair fragment was further characterised after digestion with various restriction enzymes by hybridisation with specific probes. In all cases the same results were obtained with the three independent clones. It was previously shown that cellular fragment a contains a DNA sequence, coding for the 3' half of the ov mRNA, physically separated from the remaining of the split

Fig. 2 Preparative gel electrophoresis of *EcoRI* digested chicken DNA. DNA was extracted from hen erythrocytes³. 1 mg of *EcoRI* digested DNA³ together with *EcoRI* fragments of ³H-labelled λ DNA were loaded on a 0.6% Agarose gel (8 cm long, loading surface: 1×15 cm). Continuous elution through a sintered glass elution chamber was essentially as described (W.R., in preparation). The flow rate of the elution buffer was about 25 ml h^{-1} and 4-ml fractions were collected. The elution pattern of the ³H- λ DNA internal markers was used to construct a calibration curve relating elution position to molecule weight¹⁷. The recovery of the radioactivity was 85–100%. DNA from the various fractions was immobilised onto nitrocellulose sheets (Schleicher and Schull BA 85) using a multiple slot filtration device, such that denatured DNA from 30 different fractions could be fixed on one sheet at a time. Strips (130 mm long and 5 mm wide) with 30 transversal spots of DNA were cut, rolled into small tubes, and hybridised with a ³²P-labelled *Hhaov* probe³ (1 to 2×10^8 c.p.m. μg^{-1} , 20 h, 68 °C, 1.5 ml containing 4.5×10^6 c.p.m., hybridisation procedure as in ref. 3). The hybridisation pattern in the region of the fragment a is shown. The total amount of DNA used for hybridisation corresponds to 15% of the DNA applied on the gel (150 μg). Fractions corresponding to the hybridisation peak were pooled and concentrated by ethanol precipitation to yield 22 μg of enriched DNA.

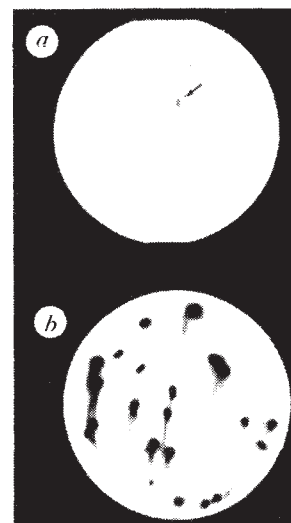
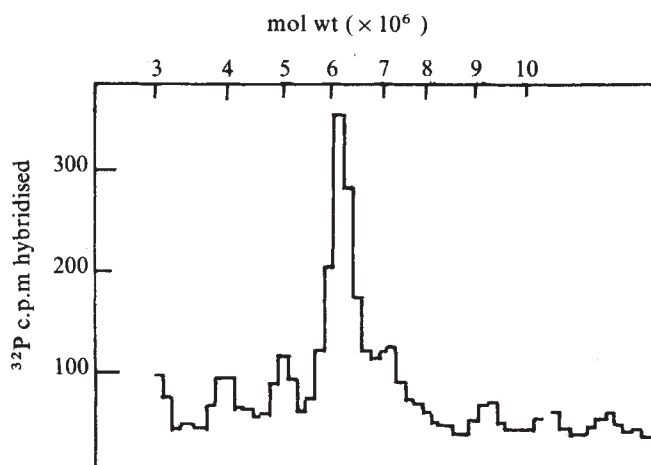


Fig. 3 *In situ* hybridisation and isolation of recombinant phages. DNA from phage λ gtWES. λ C was digested with *EcoRI* and the left and right arms were purified by preparative gel electrophoresis (as in Fig. 2) without any loss of biological activity. The recombination mixture contained 1.2 μg of left arm, 0.8 μg of right arm, and 0.5 μg of enriched DNA (molar ratio, vector: DNA = 1:2), and T4 DNA ligase (60 units) in a final volume of 25 μl (ref. 20). The reaction was carried out overnight at 4 °C. The recombinant phages were then packaged *in vitro* as described by B. Hohn¹⁹. The induced bacteria were exposed to ultraviolet light so that no more than one phage in 10^7 phages would survive. 50 μl of extract encapsidate 1 μg of exogenous intact phage DNA to produce 2×10^7 phages with a background of endogenous λ imm 434 phages of less than 10^3 phages. 2×10^5 to 4×10^5 recombinant phages were formed per μg of vector. With no other DNA added the vector preparation generated very few viable particles (less than 10^3 per μg of vector), so that more than 99.9% of phages formed *in vitro* were recombinant phages. Phages were plated on strain 803 r⁻ λ cm⁻ supE supF (ref. 21). The *in situ* hybridisation method was derived from our earlier observation that phage DNA within lysis plaques can be transferred onto nitrocellulose filters and is able to hybridise²². Our method is essentially the same as that of Benton and Davis²³. Phages are plated on L plates (about 3,000 phages per plate). DNA within the plaques is adsorbed onto nitrocellulose filters (Schleicher and Schull, BA 85) laid on the plates for 15 min. The filters are then exposed for 30 s to 0.1 M NaOH, 1.5 M NaCl, washed for 20 s, first in 0.2 M Tris, pH 7.5, 0.002 M EDTA, then in $2 \times \text{SSC}$, 0.002 M EDTA, 0.025 M phosphate buffer, pH 7.2, and dried for 2 h at 80 °C. The filters are then preincubated for 4 h at 68 °C in $6 \times \text{SSC}$, $1 \times$ Denhardt solution²⁴, then hybridised for 12 h at 68 °C in $2 \times \text{SSC}$, $1 \times$ Denhardt solution, 0.002 M EDTA, 0.025 M phosphate buffer, pH 7.2, 0.5% SDS. There was 3 ml of this mixture and 450,000 c.p.m. of denatured ³²P *Hhaov* probe (1 to 2×10^8 c.p.m. μg^{-1}) per filter. Several filters were incubated together with gentle shaking. Filters were then washed four times for 45 min in $2 \times \text{SSC}$, $1 \times$ Denhardt solution, 0.5% SDS, then 1 h in $2 \times \text{SSC}$, 50% formamide, 0.5% SDS, then a few minutes in the same buffer, followed by a few minutes in 50% formamide, $0.5 \times \text{SSC}$, 0.5% SDS, and finally in $2 \times \text{SSC}$. Autoradiography was done with flash-activated Kodak Royal X-O Mat films²⁵ and intensifying screens (Hi-plus Dupont de Nemours) overnight at -70 °C. When a spot was observed, the corresponding area on the master plate (kept at 4 °C for 2 d) was picked and resuspended in broth for amplification. The isolation of phage D is shown: a, plate with 3,000 phage plaques. The arrow shows the spot(s) which in b produced about 20 intense spots among about 70 plaques on the plate.

ovalbumin gene³. The results presented in Fig. 5 (lanes 1 and 2) show that the cloned fragment a hybridised with the ovalbumin gene probe *HaeIII* A (see Fig. 1a and b) which is specific for the 3' half of the mRNA. In contrast, no hybridisation was found with the probes *HaeIII*-*PstI* and *PstI*B which are specific for the 5' part of the mRNA (see Fig. 1a and b). In the same conditions, these probes hybridised with *Hhaov* DNA (not shown).

The location of the ov mRNA-coding region seems to be similar in the cloned *EcoRI* fragment and in the cellular fragment a. This was shown by analysis of *EcoRI*/*HindIII*, *EcoRI*/*PstI* and *EcoRI*/*BamHI* digests of the cloned DNA

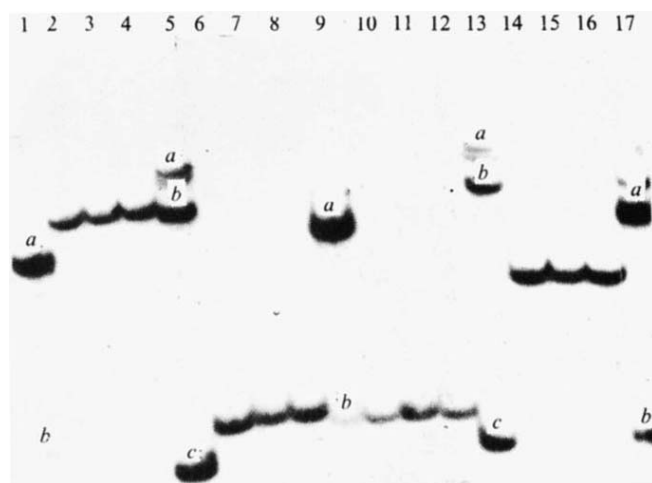


Fig. 4 Analysis of the *EcoRI* cellular DNA fragment a cloned in λ gtWESAC by digestion with restriction enzymes. DNA was purified from phages B, C and D. DNA samples (about 0.5 μ g) were digested to completion with *EcoRI*, *EcoRI* plus *PstI*, *EcoRI* plus *HindIII* or *EcoRI* plus *BamHI*, and electrophoresed on 1% Agarose slab gels (17 cm long, 2 mm thick, lane width 8 mm) at 2 V cm^{-1} . (For the source of the various restriction enzymes, see ref. 3.) The DNA fragments were transferred onto nitrocellulose filters and hybridised with ^{32}P *Hhaov* labelled by nick-translation³. After washing, the hybrid molecules were revealed by autoradiography as previously described³. Lane 2-4, *EcoRI* digests of phages B, C and D DNA, respectively. The size of the fragment which gives rise to hybridisation is 9,200 base pairs. Lanes 6-8, *EcoRI*+*PstI* digests of phage B, C and D DNA, respectively. The size of the hybridising fragment is 2,750 base pairs. Lanes 10-12, *EcoRI*+*HindIII* digests of phage B, C and D DNA, respectively. The size of the hybridising fragment is 2,600 base pairs. Lanes 14-16, *EcoRI*+*BamHI* digests of phage B, C and D DNA, respectively. The size of the hybridising fragment is 4,600 base pairs. Lanes 1, 9 and 17, internal markers for molecular weight and hybridisation, prepared from pCR1 ov2.1 DNA cleaved with various restriction enzymes (see ref. 3). The size of the fragments are (in kilobase pairs): a, 6.5; b, 2.6. Lanes 5 and 13, internal markers; size (in kilobase pairs): a, 13.5; b, 8.3; c, 2.4.

(Fig. 4). Single *EcoRI*/*PstI* (lanes 6-8), *EcoRI*/*HindIII* (lanes 10-12) and *EcoRI*/*BamHI* (lanes 14-16) bands were revealed after hybridisation with labelled *Hhaov* DNA. Their lengths (2,750, 2,600 and 4,600 base pairs, respectively) were those expected from the map previously established for the cellular *EcoRI* fragment a (ref. 3 and J. L. Mandel, unpublished).

The following results indicate that the ov mRNA-coding DNA sequence is split at the same place in the cloned fragment a as in its cellular counterpart. The interruption was previously located between the *Hinf2* and *HaeIII* sites of the ovalbumin ds-DNA (see ref. 3 and Fig. 1a and b). This was inferred from the absence of fragment *HinfA* (800 base pairs, see Fig. 1) in an *HinfI* cellular DNA digest where it was replaced by a 800-base pair fragment. The latter was not cleaved by *HaeIII* (ref. 3) which cuts once in fragment *HinfA* (see Fig. 1), indicating that the interruption in the ov mRNA-coding sequence is located to the right of the *HaeIII* site (Fig. 1). As shown in Fig. 5 (lane 3-4), the *HinfA* band was also missing in the *HinfI* digest of the cloned DNA and was replaced by a band of 800 base pairs. As expected, this 800-base pair fragment was not cut by *HaeIII* (lane 6), whereas the *HinfA* fragment from *Hhaov* DNA was cut (lane 5) in the same conditions.

The above results, summarised in Fig. 1, suggest that the *EcoRI* DNA fragment integrated in all three clones is the cellular *EcoRI* fragment a which contains a segment of the split ovalbumin gene coding for the 3' half of the ov mRNA. Therefore, the combination of *in vitro* packaging and *in situ* hybridisation is an efficient method for cloning a single-copy DNA fragment once it has been purified in a single step to a limited extent

(about 50-fold). This is shown by the fact that the desired recombinants were obtained with the expected frequency. As the same copy of the cellular DNA fragment a was present in the three independent clones analysed, it is unlikely that any large rearrangement has occurred during cloning. Therefore, the entire procedure seems to be reliable. In fact, in our procedure, the cloning of a unique cellular DNA fragment can be achieved with 10-50 ng of enriched DNA and can be used to clone any *EcoRI* DNA fragment (within certain size limits) for which a radioactive probe is available. Different procedures have been independently developed to isolate mouse immunoglobulin²⁸ and globin²⁹ genes.

Location and orientation of the ovalbumin mRNA-coding sequence in the cloned DNA fragment

Previous studies have indicated that the approximately 1,000-base pair region of the split ovalbumin gene which codes for the 3' half of the ov mRNA should be located close to one end of the *EcoRI* fragment a, within the 2,600-base pair *EcoRI*-*HindIII* fragment (see Fig. 6b in ref. 3). The availability of the cloned *EcoRI* fragment a now allows the direct determination by electron microscopy of the exact location of these ov mRNA-coding sequences and of their orientation.

Pure ov mRNA was hybridised with the purified cloned fragment a in conditions where DNA-RNA hybrids were readily formed, whereas reannealing of the DNA strands was prevented³⁰. Figure 6 shows such a typical hybrid molecule. A unique and thicker DNA-RNA hybrid region located at the end of a long single-stranded DNA stem is linked to two single-stranded branches. Sixty-seven molecules were measured and the results are represented schematically in Fig. 1c. The length of

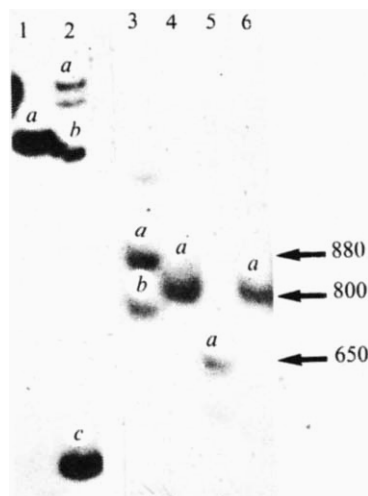


Fig. 5 Hybridisation of cloned DNA digested with *EcoRI*, *HinfI* and *HinfI*/*HaeIII* endonucleases with the *HaeIII*A probe. Phage B DNA and the *Hhaov* fragment were digested with *EcoRI*, *HinfI* or *HinfI* plus *HaeIII* as described in ref. 3. After Agarose gel electrophoresis (1% for lanes 1 and 2, 1.5% for lanes 3-6) and transfer onto nitrocellulose filters³, hybridisation was carried out with a ^{32}P -labelled *HaeIII*A probe prepared as described in the legend to Fig. 3 of ref. 3. Autoradiography was carried out as previously described³. Lane 1, *EcoRI* digest of phage B DNA, band a size 9,200 base pairs. Lane 4, *HinfI* digest of phage B DNA, band a size 800 base pairs. Lane 6, *HinfI* plus *HaeIII* digest of phage B DNA, band a size 800 base pairs. Lane 2, internal markers for molecular weight obtained as described in the legend to Fig. 4. Sizes: band a, 13,500 base pairs; band b, 8,300 base pairs; band c, 2,400 base pairs. Lane 3, internal markers, bands a and b correspond to the *HinfA* and *HinfB* fragments of *Hhaov* (Fig. 1a and ref. 3), 880 and 750 base pairs long, respectively. Lane 5, internal marker, band a (650 base pairs), obtained by double digestion of *Hhaov* with *HinfI* plus *HaeIII* (Fig. 1a and ref. 3).

the double-stranded RNA-DNA hybrid was $1,080 \pm 50$ base pairs, whereas the length of the single stranded stem was $7,800 \pm 550$ nucleotides, and those of the two single-stranded branches were $1,902 \pm 43$ and 921 ± 55 nucleotides.

After RNase A treatment the 921-nucleotide-long branch disappeared, indicating that it corresponds to the non-hybridised 5' half of the ov mRNA. In addition, a non-hybridised tail, apparently shorter than 50 nucleotides, was often seen at the unbranched end of the DNA-RNA hybrid (Fig. 6). This tail was not removed by RNase A treatment and very likely corresponds to the 3' poly A sequence of the mRNA. As no loop was observed in the DNA-RNA hybrid region, it is likely that there is no interruption in the 1,080-base pair DNA sequence which codes for the 3' half of ov mRNA. This conclusion was further

Fig. 6 Location and orientation of the ovalbumin mRNA within the cloned fragment a. *a*, Electron micrograph of a single strand of cloned purified fragment a hybridised to ov mRNA. Pure ov mRNA obtained by preparative electrophoresis of oligo-dT-cellulose purified oviduct mRNA (A.K., M. Lemeur and P. Gerlinger, in preparation) was hybridised in conditions where DNA-RNA hybrids are more stable than duplex DNA^{12,13,30}. DNA ($3 \mu\text{g ml}^{-1}$ of purified fragment a, see legend to Fig. 7) and ov mRNA ($3 \mu\text{g ml}^{-1}$) were mixed in 70% deionised formamide, 0.3 M NaCl, 0.01 M Tris-HCl, pH 8.5, 0.001 M EDTA, heated for 5 min at 75 °C to denature the DNA and incubated for 3 h at 58 °C (about 10 °C above the T_m of duplex DNA, M. Cochet, personal communication). Immediately following incubation the hybrid mixture was diluted 20- to 30-fold with spreading buffer and spread as described in ref. 31. The film was picked up onto nitrocellulose-coated grids stained with uranyl acetate (10^{-3} M in 90% ethanol) and rotary shadowed with platinum. The samples were visualised and photographed at a magnification of 18,500 with a Philips 301 electron microscope. Length measurements (at a total magnification of 137,000) were made directly on a TV monitor coupled to the electron microscope, with the help of a Digistrand M 370 P Matra facing the video screen and connected to a 980 A Texas Instrument computer. The magnification was determined before and after each series of measurement with the help of a grating replica N 1002 (Fullam). Single stranded ΦX174 DNA and double stranded ΦX174RF DNA were used as internal length standards and it was assumed that the mass per unit length of a DNA-RNA hybrid and of single stranded RNA were the same as those of double stranded DNA and single stranded DNA, respectively. The single arrows point to the two ends of the RNA-DNA hybrid region (H). The double arrow indicates the poly A tail (A). R, single stranded RNA branch; D, single stranded DNA branch; S, single stranded DNA stem. The bar represents 0.2 μm . *b*, Electron micrograph of an R-loop prepared from cloned purified fragment a and ov mRNA³². R-loops were formed in 70% deionised formamide, 0.1 M PIPES, pH 7.4, 0.1 M NaCl, 10 mM EDTA in a total volume of 30 μl containing about $3 \mu\text{g ml}^{-1}$ of DNA and $3 \mu\text{g ml}^{-1}$ of ov mRNA. The incubation was for 7-14 h at 47 °C (optimum temperature for R-loop formation according to M. Cochet, personal communication). At the end of the incubation period the samples were processed for electron microscopic examination as described above. Note the branch corresponding to the non-hybridised 5' half of the ov mRNA at one end of the hybrid region (A); the DNA-RNA hybrid region of the R-loop (B); the displaced single stranded DNA strand (C); and the 3' end of the ov mRNA (D). The bar represents 0.2 μm .

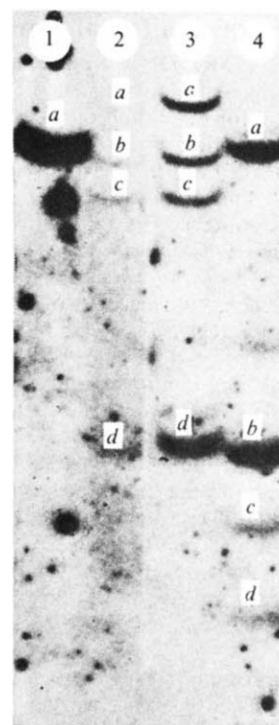
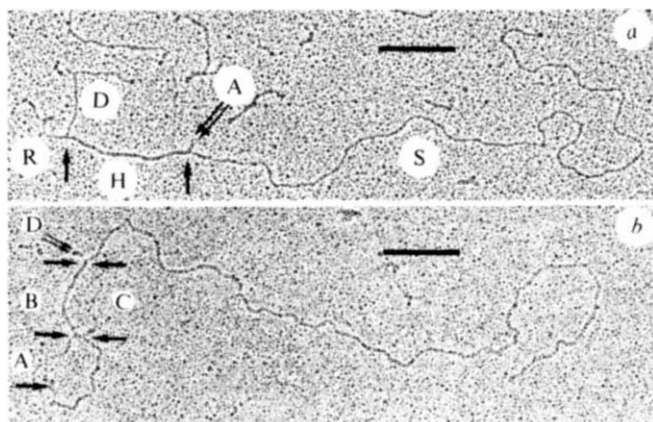


Fig. 7 Detection of fragments containing sequences complementary to the cloned fragment a in *EcoRI* digest of chicken DNA. DNA samples were prepared, electrophoresed (0.7% Agarose gel), transferred onto nitrocellulose filters, hybridised to nick-translated ^{32}P -labelled cloned fragment a or to ^{32}P -labelled *Hhaov*, and the hybrids were revealed by autoradiography as described in the legend to Fig. 4. Pure cloned fragment a was obtained by Agarose gel electrophoresis (1%) of an *EcoRI* digest of phage B DNA. Lanes 1 and 2, hybridisation to ^{32}P cloned fragment a, lanes 3 and 4, hybridisation to ^{32}P *Hhaov*. Lane 1, *EcoRI* digest of chicken DNA. Size of fragment a (in kilobase pairs) 9.2. Lane 4, *EcoRI* digest of chicken DNA. Size of the fragments (in kilobase pairs): a, 9.2; b, 2.35; c, 1.8; d, 1.3. Lanes 2 and 3, internal markers for molecular weight prepared from pCR1ov2.1 DNA (see ref. 3). Size of the fragments (in kilobase pairs): a, 13.5; b, 8.3; c, 6.5; d, 2.4.

supported by the finding that the nuclease S1 resistant hybrid formed between ovalbumin cDNA and the cloned DNA fragment a is about 1,050 base pair long (C. Benoist and R.B., unpublished).

The orientation, within the cloned DNA fragment a, of the DNA sequence coding for the 3' half of the ov mRNA was given both by the position of the 5' half of the ov mRNA which did not hybridise to the cloned DNA fragment and by the position of the poly A tail (Fig. 6). As illustrated in Fig. 1c, the DNA sequence coding for the 3' half of the mRNA is located approximately between position 1,100 and 2,150 within the 2,600-base pair *EcoRI/HindIII* fragment. Position 2,150 corresponds to the 3' end of the ov mRNA, whereas position 1,100 is the region where the ov mRNA-coding DNA sequence is interrupted. Therefore, the direction of transcription of the ov mRNA-coding DNA sequence carried by fragment a must be from left to right on the map drawn in Fig. 1c. Similar conclusions could be drawn from the analysis of R-loop molecules^{13,32} formed between fragment a and pure ov mRNA (Fig. 6b).

When R-loops were formed with intact recombinant phage DNA, rather than with fragment a, two types of molecules were observed depending on which phage clone was used (not shown). For clones B and D, the R loop was situated in the middle of the molecule, whereas for clone C, the R loop was located at about one-third from one end of the molecule. These results indicate that the insertion of fragment a has occurred in the two possible orientations and that both types of phage are viable.

Organisation of the split ovalbumin gene

The cloning of an *EcoRI* DNA fragment exhibiting all the characteristics of cellular DNA fragment a (ref. 3) and carrying only the information for the 3' half of the ov mRNA definitely proves the split organisation of the ovalbumin gene.

Two types of model have been proposed for the structural organisation of the split ovalbumin gene³. In one model, the insertion model, the different regions coding for the ov mRNA are all on the same DNA segment in the same order and orientation as in the *in vitro* synthesised ds-cDNA, but they are separated by intervening sequences (also termed insertions) not represented in the ov mRNA. In the second model the different regions are not necessarily in the same order and orientation as in the ds-cDNA. Although our present results do not allow us to distinguish between the two models, they lead to further predictions concerning the insertion model³. From the orientation of the ov mRNA-coding sequences shown in Fig. 1c, the other regions of the split gene should be located to the left of the leftward *EcoRI* site. Therefore, in the insertion model, the minimum length of the intervening sequence separating the ov mRNA-coding sequence contained in fragment a and the nearest ov mRNA-coding region must be at least the 1,100 base pairs located to the left of the ov mRNA-coding sequence (see Fig. 1c).

To investigate if this putative intervening sequence is repeated elsewhere in the chicken genome, we have prepared a highly labelled cloned fragment a and used it to monitor the distribution of complementary sequences within an *EcoRI* digest of chicken DNA. As shown in Fig. 7 (lane 1) the only band which hybridised was fragment a itself, whereas in a parallel experiment (lane 4) the *HhaI* probe revealed all four *EcoRI* fragments of the ovalbumin gene³. It seems, therefore, that the putative insertion region is not present in other fragments of the genome and, more generally, that fragment a contains no extensive sequence repeated elsewhere in the chicken genome. Finally, these results, as well as the isolation of three independent clones bearing the same fragment a, strongly suggest that there is only one type of organisation for this part of the split ovalbumin gene in the chicken genome.

Biohazards associated with the experiments described here have been examined by the French National Control Committee.

The experiments were carried out according to L3 B2 conditions in the nomenclature adopted by the French Committee³³ (L3 B2 is considered equivalent to P3 EK2 in NIH nomenclature). We thank Dr S. Tonegawa for communicating unpublished results and procedures, Drs B. Hohn and L. Enquist for the gift of strains and Drs F. Rougeon, A. Fritsch, M. Le Meur, M. Cochet, P. Gerlinger, F. Gannon and J. L. Mandel for the materials. This work was supported, in Paris, by grants to P.K. from CNRS (ERA 201, plus an exceptional grant), DGRST, and INSERM (CRAT 76.4.465) and, in Strasbourg, by grants to P.C. from INSERM (CRT 75.468 and 76.5.462), CNRS (ATP 2117) and the Fondation pour la Recherche Médicale Française. W. R. and J.P.L. were supported by DGRST fellowships, and R.B. by an EMBO fellowship.

Received 3 January; accepted 22 March 1978.

1. Sambrook, J. *Nature* **268**, 101–104 (1977).
2. Chambon, P. *Cold Spring Harb. Symp. quant. Biol.* **42** (in the press).
3. Breathnach, R., Mandel, J. L. & Chambon, P. *Nature* **270**, 314–319 (1977).
4. Doel, M. T., Houghton, M., Cook, E. A. & Carey, N. H. *Nucleic Acids Res.* **4**, 3701–3713 (1977).
5. Williamson, B. *Nature* **270**, 295–297 (1977).
6. Jeffreys, A. J. & Flavell, R. A. *Cell* **12**, 1097–1108 (1977).
7. Leder, P. *et al. Cold Spring Harb. Symp. quant. Biol.* **42** (in the press).
8. Tilghman, S. M. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
9. Brack, C. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5652–5656 (1977).
10. Glover, D. M. & Hogness, D. S. *Cell* **10**, 167–176 (1977).
11. Pellegrini, M., Manning, J. & Davidson, N. *Cell* **10**, 213–224 (1977).
12. Wellauer, P. K. & Dawid, I. B. *Cell* **10**, 193–212 (1977).
13. White, R. L. & Hogness, D. B. *Cell* **10**, 177–192 (1977).
14. Goodman, H. M., Olson, M. V. & Hall, B. D. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5453–5457 (1977).
15. Valenzuela, P., Venegas, A., Weinberg, F., Bishop, R. & Rutter, W. J. *Proc. natn. Acad. Sci. U.S.A.* **75**, 190–194 (1978).
16. Humphries, P. *et al. Nucleic Acids Res.* **4**, 2389–2406 (1977).
17. Pacht, C. A. & Young, E. T. *Proc. natn. Acad. Sci. U.S.A.* **73**, 312–316 (1976).
18. Enquist, L., Tiemeier, D., Leder, P., Weisberg, R. & Sternberg, N. *Nature* **259**, 596–598 (1976).
19. Hohn, B. & Murray, K. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3259–3263 (1977).
20. Kourilsky, P. *et al. Biochimie* (in the press).
21. Murray, N. E., Brammar, W. J. & Murray, K. *Molec. gen. Genet.* **150**, 53–61 (1976).
22. Sanzey, B., Mercereau, O., Ternynck, T. & Kourilsky, P. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3394–3397 (1976).
23. Benton, W. P. & Davis, R. W. *Science* **196**, 180–182 (1977).
24. Denhardt, D. *Biochem. biophys. Res. Commun.* **23**, 641–646 (1966).
25. Laskey, R. A. & Mills, A. D. *FEBS Lett.* **82**, 314–316 (1977).
26. Leder, P., Tiemeier, D. & Enquist, L. *Science* **196**, 175–177 (1977).
27. Southern, E. M. *J. molec. Biol.* **98**, 503–518 (1975).
28. Tonegawa, S., Brack, D., Hozumi, N. & Schuller, R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3518–3522 (1977).
29. Tilghman, S. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 4406–4410 (1977).
30. Casey, J. & Davidson, N. *Nucleic Acids Res.* **4**, 1539–1552 (1977).
31. Davis, R. W., Simon, M. & Davidson, N. *Meth. Enzym.* **21 D**, 413–428 (1971).
32. Thomas, M., White, R. L. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2294–2298 (1976).
33. *Progrès Scientifique* No. 191 (November–December 1977).

A mutation affecting the σ subunit of RNA polymerase changes transcriptional specificity

Andrew A. Travers & Robin Buckland

MRC Laboratory of Molecular Biology, Cambridge, UK

M. Goman, S. S. G. Le Grice & J. G. Scaife

Department of Molecular Biology, University of Edinburgh, Edinburgh, UK

The RNA polymerase mutation, alt-1, affects the σ subunit and alters the in vitro selectivity of RNA polymerase to parallel the in vivo phenotype. We propose that the mutation changes the distribution of functionally distinct polymerase isomers.

TRANSCRIPTION of the catabolic sensitive operons in *Escherichia coli* normally requires both cyclic AMP (cAMP) and the crp protein *in vivo*^{1,2} and *in vitro*^{3–5}. Strains lacking either crp protein (*crp*[−]) or adenyl cyclase (*cya*[−]) are unable to initiate transcription of these operons at a high rate. By

selecting mutants of a *cya*[−] strain that could utilise arabinose as the sole carbon source Silverstone *et al.*⁶ defined a class of mutation, termed *alt*, which compensates for the loss of the cAMP–crp system. The *alt* mutants isolated in this way efficiently express other catabolite sensitive operons in addition to the *ara* operon but do not contain enhanced levels of cAMP. Further, the mutants are temperature sensitive both for growth on any medium and for the expression of at least the *lac*⁶ and *ara* (M. G. and J. G. S., unpublished results) operons. At the restrictive temperature (42 °C) the overall pattern of RNA synthesis is grossly changed, and consequently, Silverstone

*et al.*⁶ suggested that these *alt* mutations might affect either a component of RNA polymerase or a transcription factor.

We show here that RNA polymerase isolated from strains carrying the mutation *alt-1* is altered both in transcriptional specificity and physical properties. This enzyme can transcribe *lac* mRNA in the absence of cAMP and *crp* protein up to six times more efficiently than RNA polymerase isolated from an isogenic *alt*⁺ strain, an effect which is apparent at low temperatures but not at high. To this extent the *alt-1* polymerase mimics the *in vivo* phenotype. In this and other respects the properties of the *alt-1* polymerase qualitatively resemble those which wild-type RNA polymerase takes on in the presence of low concentrations of the nucleotide ppGpp.

The biochemical data show that the change in transcriptional specificity is a consequence of an alteration of the σ subunit of RNA polymerase. These mutants thus confirm the previous biochemical observations that σ can act as a specificity determinant in promoter selection⁵⁻⁹. Our genetic results show that *alt-1* maps close to *dnaG* on the *E. coli* chromosome, thus providing independent evidence for the provisional location for the structural gene for σ at 66 min (refs 10, 11).

Altered properties of RNA polymerase from *alt-1* strains

Mutant RNA polymerases commonly show an increased inhibition of RNA synthesising activity at high salt concentrations¹²⁻¹⁵. To test whether the *alt-1* mutation directly affects RNA polymerase activity we measured the ionic strength dependence of transcription by purified enzyme from the mutant WZ 30 (Table 1) and its isogenic parent WZ 22. Figure 1a shows that when equal weights of the enzyme from the *alt*⁺ and *alt-1* strains are assayed with ϕ 80 DNA, transcription by polymerase from WZ 30 is con-

siderably more salt sensitive than that from WZ 22.

Conceivably, such a change in properties could be unrelated to the original *alt-1* mutation. RNA polymerase was therefore isolated from two transductants, differing only at the *alt* locus (Table 1). Again, the enzyme from the *alt-1* transductant, WZ 301, was more salt sensitive than that from the *alt*⁺ transductant, WZ 300. We conclude that this increased salt sensitivity of the enzyme is associated with the *alt-1* mutation.

A second distinguishing property of RNA polymerase from *alt-1* strains is its characteristic sedimentation profile. When RNA polymerase from WZ 22 is sedimented through a 15–30% glycerol gradient containing 0.2 M KCl the enzyme activity sediments as a broad peak with an average S value of ~14 S (Fig. 2a). By contrast, that from WZ 30 sediments as a sharper peak at ~13.5 S. The difference in sedimentation profiles is not a consequence of the presence of inactive enzyme in the WZ 30 preparations, as in both cases the enzyme activity parallels the amounts of β and β' subunits present (data not shown).

Analysis of the sedimentation profiles of RNA polymerase isolated from WZ 300 and WZ 301 reveals the same difference in properties (Fig. 2b). Thus, both the increased salt sensitivity of ϕ 80 DNA transcription and altered sedimentation characteristics correlate with the presence of the *alt-1* mutation.

alt-1 RNA polymerase has altered σ activity

To determine which component of the WZ 30 RNA polymerase was responsible for the alteration of enzyme activity, RNA polymerase from WZ 22 and WZ 30 was chromatographed on Biorex 70 to separate the σ subunit from the core polymerase. Holoenzymes were reconstituted using homologous and heterologous core and σ components and assayed with ϕ 80 DNA as the template. With the core polymerase from WZ 22, the enhanced salt sensitivity of

Table 1 Mapping of *alt*: cotransduction with *tolC*

Relevant genotype Donor	Recipient	Marker selected	Expt no.	Total transductants	No. tested	Test for ts No. ts	%	Coincidence of ts and ara ⁺ ts ⁺	ts ⁻
1. WZ-22 <i>cya-855 alt</i> ⁺	WZ-98 <i>cya-855 alt</i> ⁺ <i>tolC</i> ⁺	<i>tolC</i> ⁺	I	16	16	0	< 6%	Ara ⁻	None
2. WZ-30 <i>cya-855 alt-1</i>	WZ-98 <i>cya-855 alt</i> ⁺ <i>tolC</i> ⁺	<i>tolC</i> ⁺	I	30	30	16	53%	Ara ⁻	Ara ⁺
	<i>metB tolC</i>		II	76	50	25	50%	Ara ⁻	Ara ⁺
3. X12004 <i>dnaG alt</i> ⁺	WZ-98 <i>cya-855 alt</i> ⁺ <i>tolC</i> ⁺	<i>tolC</i> ⁺	I	35	35	19	54%	Ara ⁻	Ara ⁻
	<i>metB tolC</i>		II	56	50	23	43%	Ara ⁻	Ara ⁻
4. X12004 <i>dnaG alt</i> ⁺	WZ-98 <i>cya-855 alt</i> ⁺ <i>metB</i> ⁺	<i>metB</i> ⁺	I	37	33	0	< 3%	Ara ⁻	None
	<i>metB tolC</i>		II	44	40	0	< 3%	Ara ⁻	None
5. WZ-11 <i>cya</i> ⁺ <i>alt</i> ⁺	X12005 <i>dnaG alt</i> ⁺ <i>tolC</i> ⁺	<i>tolC</i> ⁺	I	50	50	26	52%	—	—
6. WZ-30 <i>cya-855 alt-1</i>	X12005 <i>dnaG alt</i> ⁺ <i>tolC</i> ⁺	<i>tolC</i> ⁺	I	25	25	25	100%	—	—
7. X12004 <i>dnaG alt</i> ⁺	X12005 <i>dnaG alt</i> ⁺ <i>tolC</i> ⁺	<i>tolC</i> ⁺	I	21	21	21	100%	—	—

All the WZ strains used here are isogenic. WZ11 F⁻ *metB relA str-r* is a spontaneous *str-r* mutant of W1655 (ref. 24). WZ22 *cya-855* is an ultraviolet radiation-induced mutant of WZ11 (ref. 6). WZ30 is the spontaneous *alt-1* revertant of WZ22 (see text). WZ98 derives from WZ22 by spontaneous mutation to 'glp⁺' (ref. 6) (not relevant to this study) and to ColE1 tolerance (*tolC*). WZ300 and WZ301 are *tolC*⁺ transductants of WZ98, bearing *alt*⁺ and *alt-1*, respectively. X12004 F⁻ *leu thy thi dnaG3* is also called PC3 (ref. 34). X12005 is a spontaneous *tolC* mutant of X12004. *TolC* strains were selected on nutrient plates previously seeded with a ColE1 strain in top agar, incubated overnight and chloroformed. Colonies surviving this selection were screened for sensitivity to phage BF23 and methylene blue (65 μ g ml⁻¹ in MS agar⁶). ColE1 resistant mutants sensitive to these agents are *tolC*²⁵. Transductions were carried out using P1 vir as vector²⁶. *tolC*⁺ transductants were selected as methylene blue resistant colonies (65 μ g ml⁻¹ dye in MS agar). Appropriate volumes of phage and recipient bacteria were plated separately on selective medium as controls for each transduction. Suspensions of purified transductant colonies were tested for temperature sensitivity by spotting 10 μ l on two nutrient plates for incubation at 30 °C and 42 °C. ts transductants plated at least 10⁴-fold less efficiently at 42 °C. Similarly, representative transductants were tested for their ability to grow on arabinose as the sole carbon source without cAMP.

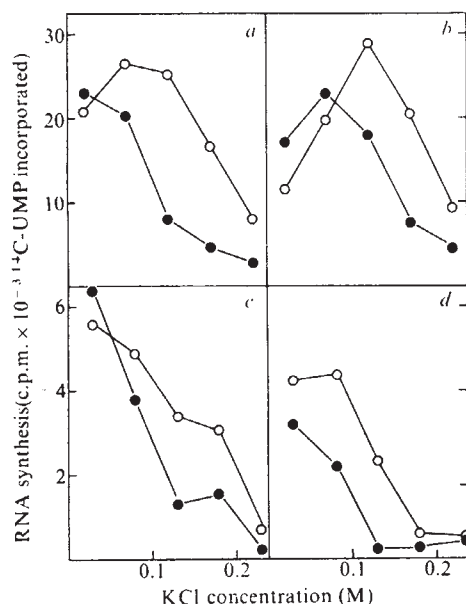


Fig. 1 Salt sensitivity of $\phi 80$ DNA transcription. RNA polymerase holoenzymes were purified from 15–30 g frozen cells by the method of Burgess and Travers²⁷ and further purified by chromatography on DNA cellulose²⁸. Polymerase so prepared was $\geq 97\%$ pure and contained 0.6–0.8 mol σ subunit per 2 mol α subunit. σ Subunit was separated from core polymerase by chromatography on Biorex 70 (ref. 28). The σ preparations used stimulated T4 DNA transcription by core polymerase by 30–45-fold and contained no impurities detectable at the 5% level by gradient gel electrophoresis in 0.1% SDS. Holoenzymes were reconstituted by the addition of saturating amounts of σ activity to the core polymerase. $\phi 80$ DNA transcription was assayed as previously described¹⁹. *a*, 10 μ g per assay holoenzymes from WZ 22 and WZ 30; *b*, 10 μ g holoenzymes from WZ 300 and WZ 301; *c*, 2 μ g core polymerase from WZ 22 combined with σ preparations from WZ 22 or WZ 30; *d*, 1.7 μ g core polymerase from WZ 30 combined with σ preparations from WZ 22 or WZ 30. In all panels, $\circ$ and $\bullet$ indicate the alt^+ and alt^- enzymes, respectively.

transcription is conferred by WZ 30 σ (Fig. 1c). Similarly, with WZ 30 core polymerase this *in vitro* characteristic of alt^- polymerase is again a property of WZ 30 σ (Fig. 1d). Thus, the alteration in the properties of RNA polymerase is independent of the source of core polymerase, and hence of the α , β and β' subunits but is conferred by the σ subunit from the mutant.

The chromosomal position of *alt-1*

Previous mapping studies⁸ located *alt-1* between 62 min and 70 min on the *E. coli* chromosome. As the mutation is not cotransduced with *argG* or *pnp* at 68 min, we reason that it lies outside the segment 66–70 min. The results presented here place *alt-1* close to *dnaG* (Fig. 3).

Prompted by recent mapping studies^{10,11}, we looked for cotransduction of *alt-1* with *tolC* (65.5 min). Phage P1 grown on alt^+ and alt^- strains is used to transduce a *tolC* mutant unable to grow on minimal medium containing methylene blue. The *tolC*⁺ transductants selected on this medium are tested for coinheritance of the temperature sensitivity phenotype of the *alt-1* mutant (Table 1; 1 and 2). Half of the *tolC*⁺ transductants arising from the *alt-1* lysate are temperature sensitive for growth on rich medium, suggesting that they have acquired *alt-1* whereas none of the *tolC*⁺ transductants from the control (alt^+) lysate is temperature sensitive.

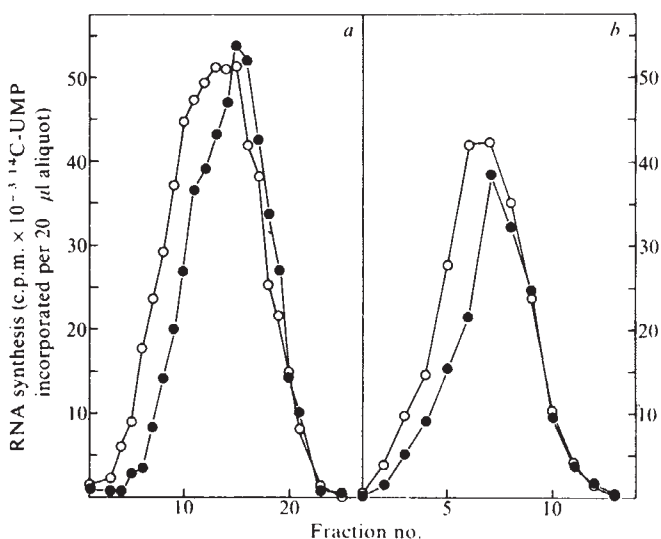
The putative *alt-1* transductants behave exactly as expected when tested for growth on arabinose as the sole carbon source. The *cya* recipient, WZ 98, can only grow on arabinose if cAMP is provided exogenously. Its *tolC*⁺ transductants, which remain *ts*⁺, retain this property (Table

1; 1 and 2). By contrast, the *ts*⁻ transductants are now able to grow on arabinose without cAMP, showing that they have acquired the cAMP-crp independence characteristic of *alt-1*. Parallel crosses show that our *tolC* mutation has normal linkage to *dnaG* (Table 1; 1, 3, 4) and that *alt-1* and *dnaG* lie close together on the same side of *tolC* (Table 1; 1, 5–7). We infer that *alt* is located at 66 min (Fig. 3), in a similar position to a gene that affects the structure of the σ subunit^{10,11}.

Selectivity and regulation of *alt-1* RNA polymerase

In vivo alt-1 leads to substantial changes in the qualitative pattern of RNA synthesis. To determine whether RNA polymerase isolated from *alt-1* strains was similarly altered in transcriptional selectivity, its ability to transcribe specific RNA species—in particular *lac* mRNA and rRNA—was tested *in vitro*. Figure 4a shows that the polymerases isolated from WZ 300 and WZ 301 differ considerably in the qualitative characteristics of *crp*-independent *lac* transcription from $\phi 80$ *plac* DNA, a template containing a wild type *lac* promoter. At 23 °C, *lac* RNA transcription by WZ 300 polymerase has a KCl optimum of 0.05 M. By contrast, the mutant enzyme from WZ 301 synthesises most *lac* RNA at 0.1–0.15 M KCl. At this concentration, total *lac* RNA synthesis by WZ 301 RNA polymerase is 3–6 times greater than that by WZ 300 enzyme. At 30 °C, these differences are no longer apparent (Fig. 4b). Indeed, WZ 301 polymerase generally synthesises less *lac* RNA than WZ 300 polymerase at this temperature. Transcription of *lac* DNA sequences by both polymerases is 80–90% inhibited by 10^{-6} M *lac* repressor at 0.11 M KCl. In these conditions, the repressor fails to inhibit synthesis from either λ promoters or from the *suII*⁺ tRNA promoter¹⁶. Similar differences in the pattern of *lac* transcription are observed

Fig. 2 Zone sedimentation of polymerase holoenzymes. *a*, WZ 22 and WZ 30 polymerases; *b*, WZ 300 and WZ 301 polymerases. About 4 mg of holoenzyme were layered on a 12 ml 15–30% glycerol gradient containing 0.01 M Tris-HCl, pH 7.9, 0.01 M MgCl₂, 0.0001 M dithiothreitol, 0.0001 M Na₂EDTA, and 0.2 M KCl. The gradients were centrifuged at 5 °C for 24 h at 35,000 r.p.m. in an MSE 40 rotor. For comparison of WZ 22 and WZ 30 polymerases 4-drop fractions were collected, giving a total of 40 fractions; for WZ 300 and WZ 301 polymerases 8-drop fractions were collected. β -Galactosidase and catalase, sedimented in a parallel gradient to WZ 22 and WZ 30 enzymes, peaked at fractions 11 and 19, respectively. Data presented are from single experiments representative of 5/5 and 2/2 preparations of the WZ 22/WZ 30 and WZ 300/WZ 301 pairs, respectively. $\circ$ and $\bullet$ represent alt^+ and alt^- enzymes, respectively.



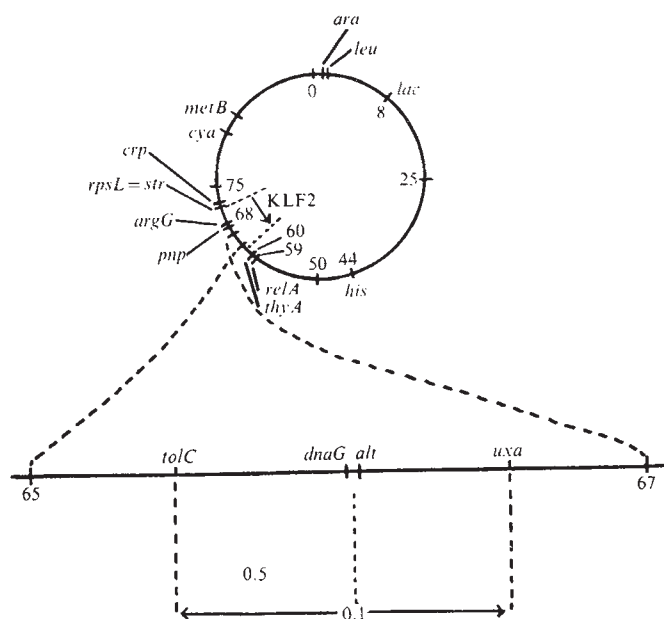


Fig. 3 Genetic map of *E. coli* showing the *alt* region. Integral numbers represent map minutes, each of which corresponds to approximately 40 kilobases. Note that the marker *dnaG3* has not been ordered with respect to *alt-1*. Genetic distances are given as average contrasduction frequencies from reciprocal crosses, that for *alt* and *tolC* is from Table 1, and that for *uxa* and *tolC* from Portalier *et al.*²⁹.

between WZ 22 and WZ 30 polymerase transcribing *lac* RNA from ϕ 80 *plac* DNA (data not shown).

Transcription of a second RNA species, rRNA, is also affected by the *alt-1* mutation. Figure 4c shows that at low KCl concentration WZ 301 polymerase synthesises more rRNA from the rRNA transcription unit carried by phage λ *d_s ilv* (ref. 17) than does WZ 300 polymerase. At high KCl concentrations this pattern is reversed. Thus, the alteration in *alt-1* polymerases can affect the transcription of

rRNA in an opposite way to *lac* RNA (compare Fig. 4a and c).

The alterations in both the salt dependence of rRNA synthesis and the sedimentation profile conferred by the *alt-1* mutation resemble the corresponding changes observed when low concentrations of the regulatory nucleotide ppGpp are added to normal wild-type RNA polymerase. To test whether these properties of the *alt-1* polymerases reflected an altered response to the nucleotide, rRNA transcription by WZ 300 and WZ 301 enzymes was assayed as a function of ppGpp concentration. Normally, two apparent association constants of 2×10^5 and 10^4 M⁻¹ characterise the functional interaction of ppGpp with RNA polymerase¹⁸. Figure 4d shows that low ppGpp concentrations, that is, up to 10 μ M, substantially increase rRNA synthesis by WZ 300 polymerase but do not affect that by WZ 301 polymerase. Higher nucleotide concentrations inhibit rRNA synthesis by both enzymes in a similar manner. We conclude that in this experiment the *alt-1* polymerase lacks the normal response to low ppGpp concentrations.

The role of σ in promoter selection

The principal conclusion from these experiments is that RNA polymerase purified to homogeneity from strains containing the *alt-1* mutation is altered both in physical properties and transcriptional specificity. The evidence suggests that *alt-1* strains contain an altered σ subunit. In particular, the enhanced salt sensitivity of ϕ 80 DNA transcription *in vitro* is conferred by the σ subunit from an *alt-1* strain. We favour the simplest interpretation of this result, that our mutation lies in the structural gene for σ (see refs 10, 11). However, our data do not at present exclude the possibility that the alteration in σ involves a post-translational modification. Another mutant, *rpoD1*, has recently been reported to affect σ function¹⁹. However, it remains to be shown whether *alt-1* and *rpoD1* lie in the same gene.

The *alt-1* mutation provides the first genetic evidence for the role of σ in promoter selection. In altering the transcriptional specificity of RNA polymerase, and thereby

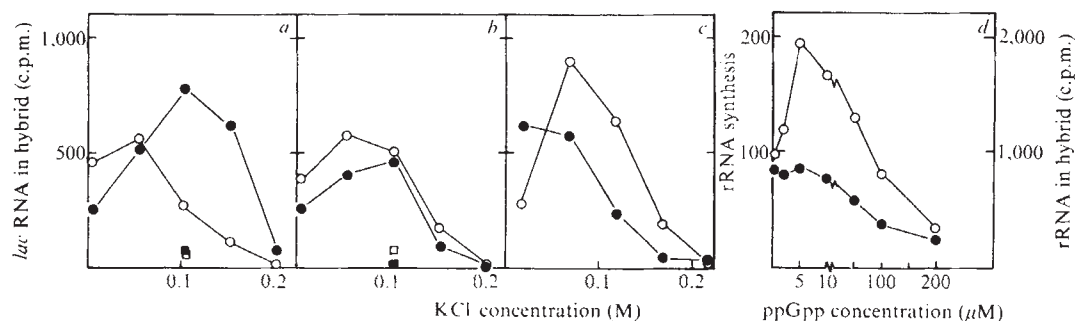


Fig. 4 *lac* RNA and rRNA synthesis by *alt*⁺ and *alt-1* polymerases. *a*, *lac* RNA synthesis by WZ 300 and WZ 301 polymerases at 23 °C; *b*, *lac* RNA synthesis by WZ 300 and WZ 301 polymerases at 30 °C; *c*, rRNA synthesis by WZ 300 and WZ 301 polymerases; and *d*, response of WZ 300 and WZ 301 polymerases to ppGpp. Reaction mixtures (200 μ l) contained

0.04 M Tris HCl, pH 7.9, 0.01 M MgCl₂, KCl as indicated, 0.006 M 2-mercaptoethanol, 0.0001 M EDTA, 0.25 mM each of ATP, CTP and GTP, 0.006 mM ³H-UTP (40 Ci mmol⁻¹), 17 μ g ml⁻¹ ϕ 80 *plac* DNA or 19 μ g ml⁻¹ λ *d_s ilv* DNA as appropriate and 10 μ g ml⁻¹ RNA polymerase holoenzyme from WZ 300 or WZ 301. All reaction mixtures were preincubated for 5 min at 23 °C (*a*) or 30 °C (*b-d*) before the addition of polymerase. RNA synthesis proceeded for 15 min and was terminated by the addition of an equal volume of 4 $\times$ SSC followed by 2 volumes 2 $\times$ SSC saturated phenol. The aqueous phase from this extraction was used as a source of RNA for hybridisation. Transcription of *lac* specific sequences from ϕ 80 *plac* DNA was determined by hybridisation of 25- μ l aliquots of extracted RNA in duplicate to 110 μ g ml⁻¹ denatured pKB 252 DNA which had been partially cleaved with *Eco*R1 nuclease³⁰. Hybridisation was for 1 h at 60 °C in 2 $\times$ SSC in a final volume of 250 μ l. Input RNA varied between 33,000 and 226,000 c.p.m. Backgrounds in the absence of DNA varied between 180 and 110 c.p.m. About 50% of the molecules in the *lac* repressor preparation were active. $\square$, $\blacksquare$ Indicate synthesis with repressor for WZ 300 and WZ 301 polymerases, respectively. $\circ$, $\bullet$ Indicate *alt*⁺ and *alt-1* enzymes, respectively. In a separate experiment no significant repression was observed in the presence of 5×10^{-4} M IPTG. The assay, in principle similar to those previously used^{4,30} measures the amount of 5'-terminal 65-nucleotide sequence of *lac* RNA³¹. The sensitivity to *lac* repressor with ϕ 80 *plac* DNA differs from that previously reported, possibly because read-through is minimised by a limiting UTP concentration³². rRNA synthesis from λ *d_s ilv* DNA was determined as described elsewhere³². For the experiment in *d* the KCl concentration was 0.05 M.

mimicking the effect of low ppGpp concentrations, the mutation shows that σ is directly involved in the process of discrimination between different promoters. By what mechanism does the mutation alter selectivity? The change in sedimentation profile consequent upon the mutation parallels the effect of 10 μ M ppGpp on normal wild-type polymerase holoenzyme¹⁸. This effect, coupled with the failure of the enzyme to respond functionally to low concentrations of ppGpp, can be explained in the context of the following model for promoter selection. Evidence to be presented elsewhere (A.A.T., R.B. and P. G. Debenham, in preparation) suggests that an RNA polymerase molecule may adopt one of several different conformations. It is proposed that each conformational isomer (conformer) has a different promoter preference. We may thus explain the present observations by supposing that the *alt-1* mutation favours the conformations which are perhaps identical to those stabilised in the wild-type enzyme by low ppGpp levels *in vitro* and which *in vivo* show a preference for catabolite sensitive promoters.

To what extent do the altered properties of the *alt-1* RNA polymerase account for the *in vivo* phenotype? *In vitro*, at low temperature (23 °C) and high monovalent cation, *alt-1* polymerase transcribes *lac* mRNA significantly more efficiently than polymerase isolated from *alt+* strains. This enhanced *lac* transcription is no longer apparent at 30 °C. The character of *in vitro lac* transcription by the *alt-1* polymerase is thus qualitatively similar to the *in vivo alt-1* phenotype, although the temperature sensitivity for *lac* RNA synthesis is observed at a lower temperature *in vitro* than *in vivo*.

The existence of an RNA polymerase mutant which compensates for *crp*-cAMP function in catabolite sensitive operon expression and which achieves this compensation by a change in specificity rather than by an enhanced affinity for all promoters, supports the proposal that *crp* protein must interact with RNA polymerase directly to increase the rate of transcription^{3,20,21}. As *crp* protein binds to DNA (refs 22, 23), one possibility is that the regulatory

protein switches the structure of RNA polymerase to the conformer appropriate for catabolite sensitive operons while both proteins are bound to the promoter region.

We thank Drs J. Robert-Baudouy, N. S. Willetts and M. Vincente for the strains, Dr N. Giesler and K. Weber for purified *lac* repressor, Dr I. B. Holland for advice, Mrs V. Ogburn and Miss H. Grozier for technical assistance and the MRC for support to J.G.S.

Received 5 December 1977; accepted 22 March 1978.

1. Perlman, R. L. & Pastan, I. *J. biol. Chem.* **243**, 5420–5427 (1968).
2. Ullman, A. & Monod, J. *FEBS Lett.* **2**, 57–60 (1968).
3. Zubay, G., Schwartz, D. & Beckwith, J. R. *Proc. natn. Acad. Sci. U.S.A.* **66**, 104–110 (1969).
4. Eron, L. & Block, R. *Proc. natn. Acad. Sci. U.S.A.* **68**, 1828–1832 (1971).
5. Crombrughe, B. de et al. *Nature new Biol.* **231**, 139–142 (1971).
6. Silverstone, M. E., Goman, M. & Scaife, J. G. *Molec. gen. Genet.* **118**, 223–234 (1972).
7. Bautz, E. K. F., Bautz, F. A. & Dunn, J. J. *Nature* **223**, 1022–1024 (1969).
8. Sugiura, M., Okamoto, T. & Takanami, M. *Nature* **225**, 598–600 (1970).
9. Hinkle, D. C. & Chamberlin, M. J. *J. molec. Biol.* **70**, 157–185 (1972).
10. Nakamura, Y., Osawa, T. & Yura, T. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1831–1835 (1977).
11. Harris, J. D., Martinez, I. I. & Calendar, R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1836–1840 (1977).
12. Jacobson, A. & Gillespie, D. *Cold Spring Harb. Symp. quant. Biol.* **35**, 85–93 (1970).
13. Georgopoulos, C. P. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2977–2981 (1971).
14. Panny, S. R. et al. *FEBS Lett.* **48**, 241–245 (1974).
15. Gross, G., Fields, D. A. & Bautz, E. K. F. *Molec. gen. Genet.* **147**, 337–341 (1976).
16. Debenham, P. G. thesis, Univ. Cambridge (1978).
17. Jorgensen, P. & Fiil, N. P. in *Control of Ribosome Synthesis* (eds Kjelgaard, N. O. & Maae, O.) 370 (Munksgaard, Copenhagen, 1976).
18. Debenham, P. G. & Travers, A. *Eur. J. Biochem.* **72**, 515–523 (1977).
19. Gross, C. et al. *Proc. natn. Acad. Sci. U.S.A.* **75**, 427–431 (1978).
20. Travers, A. A., Kamen, R. I. & Schleif, R. F. *Nature* **221**, 1012–1014 (1970).
21. Gilbert, W. in *RNA polymerase* (eds Chamberlin, M. J. & Losick, R.) 193 (Cold Spring Harbor Laboratory, New York, 1976).
22. Majors, J. *Nature* **256**, 672–674 (1975).
23. Mitra, S., Zubay, G. & Landy, A. *Biochem. biophys. Res. Commun.* **67**, 857–863 (1975).
24. Lederberg, E. & Lederberg, J. *Genetics* **38**, 51–64 (1953).
25. Holland, I. B. & Threlfall, E. J. *J. Bact.* **97**, 91–96 (1969).
26. Miller, J. H. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, New York, 1972).
27. Burgess, R. R. & Travers, A. A. in *Proc. Nucleic Acid Research 2* (eds Cantoni, C. L. & Davies, D. R.) 851 (Harper and Row, New York, 1971).
28. Burgess, R. R. & Jendrisak, J. J. *Biochemistry* **14**, 4634–4638 (1975).
29. Portatier, R. C., Robert-Baudouy, J. M. & Nemoz, G. M. *Molec. gen. Genet.* **128**, 301–319 (1974).
30. Nakanishi, S., Adhya, S., Gottesman, M. & Pastan, I. *J. biol. Chem.* **250**, 8202–8208 (1975).
31. Backman, K., Ptashne, M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **73**, 4174–4178 (1976).
32. Travers, A. *Molec. gen. Genet.* **147**, 225–232 (1976).
33. Wu, R., Banbara, R. & Jay, E. *Crit. Rev. Biochem.* **2**, 455–512 (1975).
34. Wechsler, J. A. & Gross, J. D. *Molec. gen. Genet.* **113**, 273–284 (1971).

A method for classification of 5' termini of retroviruses

William A. Haseltine

Sidney Farber Cancer Institute, Harvard Medical School/Department of Pathology, Boston, Massachusetts 02115

Dennis G. Kleid

Bio-organic Chemistry Department, Stanford Research Institute, Menlo Park, California 94025

A new method for the classification of retroviruses is presented. The scheme is based on the length and sequence of a DNA transcript of the 5' end of the genome. The method can be used to detect similarities between distantly related viruses as well as to discriminate between very closely related viruses. The method is applied to viruses isolated from mice, baboons, gibbons, a woolly monkey and chickens.

RETROVIRUSES (RNA tumour viruses) have been isolated from several species including primates, rodents, birds, pigs and

cats. The viruses may be transmitted horizontally amongst a species or may be transmitted vertically as inherited proviral genomes. There is some evidence that the viruses endogenous to one species may infect other species, and that in the course of evolution viruses originally endogenous to one species have become established in the germ line of another species (see ref. 1 for a review of these problems).

The biology of retroviruses makes it of interest to quantitatively define the relationships amongst these viruses. Here we propose a new method for such analyses. This method defines both groups of related viruses and discriminates between very closely related viruses.

Table 1 Retroviruses viruses classified by the length of the strong stop DNA

Group I: 135 ± 5
Murine viruses: Mo-MuLV, AKR (AKV-1, AKV-2), N-tropic BALB/c virus, B-tropic BALB/c virus Ki-MuLV, NZB xenotropic virus Woolly gibbon viruses: SSAV, gibbon brain isolates (GBR-1, GBR-2) GALV-San Francisco Feline viruses: Rickard FL-422, FeLV Gardener, FeLV-Theilen
Group II: 120 ± 5
Endogenous baboon viruses: M-7, M-28, 455K, BILN, PP-1 Endogenous feline viruses: RD114
Group III: 101 ± 2
Chicken viruses: RSV PrB, PrC, RSV PrBtd, AMV, RAV-0, RAV-1, RAV-60 (RAV-1)
Group IV: 150 ± 5
Mouse mammary tumour virus: GR

The length of the strong stop DNA of each virus listed was determined by analysis of the DNA products of four reactions on 20% denaturing polyacrylamide gels⁸. In one reaction, the limiting dNTP concentration was 100 μ M [α -³²P]dCTP (10 Ci mmol⁻¹). The only major product of these reactions was the strong stop DNA sequence (see Fig. 1). The limiting concentration of the nucleotide triphosphates in the other three reactions was 10 μ M and was either [α -³²P] dCTP, [α -³²P]dGTP or [α -³²P]dTTP. A major DNA product was identified and found to be present in each of these reactions, regardless of which of the dNTP's was limiting. This product was of the same length as the major DNA species synthesised when the limiting dNTP concentration was 100 μ M for each of the viruses listed, and it is this species that is called strong stop DNA. The feline leukemia viruses were those previously described⁵⁶⁻⁵⁸. The mouse mammary tumour virus was purified from GR cells. The origin of the other viruses is listed in Tables 2-5.

The method is based on the analysis of the DNA products synthesised from the extreme 5' portion of the genome. When genomic RNA is used as a template for the reverse transcriptase *in vitro*, DNA synthesis is initiated with a tRNA primer that is annealed to a unique site near the 5' end of the genome^{2,3}. The initial DNA product synthesised is a run-off product, that is, a copy of the RNA between the tRNA binding site and the 5' end of the genome. The site of synthesis of the run off product (called strong stop DNA) has been rigorously demonstrated for the Rous sarcoma virus (RSV)^{4,5}, avian myeloblastosis virus⁶ and Moloney murine leukemia virus (Mo-MuLV)⁷. These experiments demonstrate that the strong stop DNA is initiated near the 5' end of the genome (101 nucleotides RSV, and 135 nucleotides Mo-MuLV) and that it terminates opposite the 2' OmG of the 5' capping structure.

The initial observation that the length of the strong stop DNA of an avian virus is different from that of a murine virus led us to investigate the possibility that length and nucleotide composition of this DNA could be used as a tool for classification and identification of all retroviruses. Accordingly, we have characterised the strong stop DNAs of several viruses with respect to these parameters.

Retrovirus classification by length of strong stop DNA

To determine the length of the strong stop DNA, the products of an *in vitro* reaction were displayed on denaturing polyacrylamide-urea gels. On such gels the mobility of the DNA species is an accurate reflection of the molecular weight⁸. In order to standardise the reaction conditions, purified AMV reverse transcriptase was used to transcribe purified viral 70S RNA. The DNA products of such reactions are identical to those synthesised in endogenous reactions using detergent disrupted virions⁴.

For the purposes of this work the strong stop species is defined as the DNA product with the following properties. (1) It is the major DNA product synthesised *in vitro* at short incubation

times (30 min) when the concentrations of the limiting deoxyribonucleotide triphosphate (dNTP) precursor is at least 100 μ M. (2) It is the longest major product of discrete length synthesised in these reaction conditions. (When 70 S RNA is used as a template only a small fraction of the total product is elongated past the 5' end of the genome). (3) The product is apparent in reactions regardless of which of the four dNTPs is limiting in the reaction.

For the viruses studied, the identification of such a DNA product was unambiguous. Formal proof that these products represent the complete transcription of the genome between the primer and the 5' end of the genome has been presented for the genomes of RSV and AMV⁴⁻⁶. Similar observations have been made for Mo-MuLV⁷.

Figure 1 illustrates the results of an experiment in which the DNA products synthesised *in vitro* were analysed on a polyacrylamide gel. The strong stop DNA can be readily identified for each reaction. Thus the length of the strong stop DNA of the viruses in lanes 1-5 is 135 ± 5; in lanes 6 and 7, 101 ± 2; in lanes 8, 9 and 10, 120 ± 5; and in lane 11, 150 ± 5. The length of the Mo-MuLV, RSV and AMV strong stop DNAs are accurately known from the DNA sequences and serve as size markers in this gel. Data from several similar experiments are presented in Table 1.

The viruses we tested may be placed into four groups by the length of the strong stop DNA. Group I, strong stop DNA of length 135 ± 5, includes the murine viruses, the woolly-gibbon

Fig. 1 Comparison of *in vitro* DNA transcription products of retroviruses: DNA was synthesised in 200 μ l reactions that contained 0.05 M Tris-HCl (pH 8.3), 0.02% Nonidet P40, 0.006 M MgCl₂, 0.005 M dithiothreitol, 1 mM each dATP, dGTP, dTTP, 100 μ M [α -³²P]dCTP (10 Ci mmol⁻¹), 100 μ g ml⁻¹ actinomycin D, 100 μ g ml⁻¹ viral 70S RNA, and 2 units of AMV reverse transcriptase. The reactions were incubated for 30 min at 43 °C. Extraction of the nucleic acids and preparation of the DNA products for gel electrophoresis after alkaline hydrolysis are as described elsewhere⁹. The samples were layered onto a 20% polyacrylamide urea gel of the type used for DNA sequence analysis⁸. Electrophoresis was for 18 hr at 1,000 V. The viruses used are described in the legends to Tables 1-5. Lane 1: murine virus Mo-MuLV grown on NIH 3T3 cells. Lane 2: murine virus Mo-MuLV grown on NRK cells. Lane 3: woolly monkey virus SSAV. Lane 4: gibbon leukemia virus GALV-San Francisco grown on Human A204 cells. Lane 5: Feline leukemia virus FeLV-Rickard from a lymphosarcoma line FL-422 grown on cultured lymphocytes. Lane 6: chicken sarcoma virus RSV PrB grown on CEF cells. Lane 7: chicken myeloblastosis virus (AMV) (plasma) virus. Lane 8: endogenous baboon virus M-7 grown on canine thymus cells. Lane 9: endogenous baboon virus M-28 grown on bat lung cells. Lane 10: feline endogenous virus RD114 grown on human rhabdomyosarcoma cells. Lane 11: mouse mammary tumour virus MMTV grown on GR mouse cells.

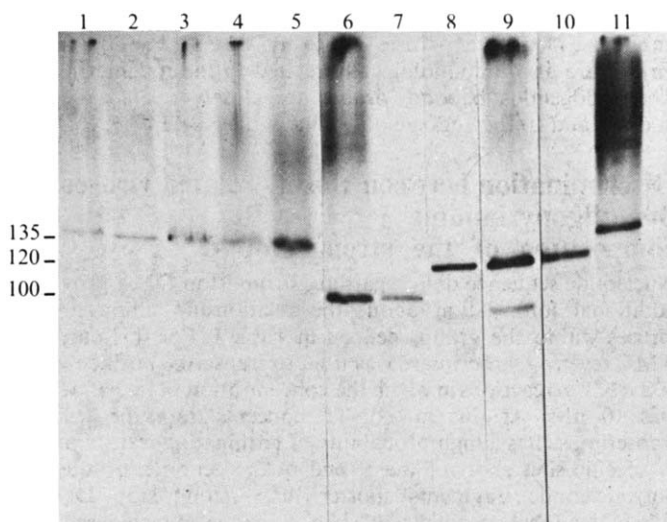


Table 2 The 5' to 3' order of the oligopyrimidines that contain dC of the murine virus strong stop DNAs

Virus	Length of strong stop DNA	Cell	5' to 3' Order of the oligopyrimidines containing dC
Subgroup I			
1, Mo-MuLV	135	NIH 3T3	(C ₃), (C, TC), (TC ₂), (TC ₆), (C ₂), (C ₃)
2, Mo-MuLV	135	NRK	
Subgroup II			
3, B-tropic Balb/c	135	NRK	(C, C ₅ , TC), (TC ₂ , T ₂ C), (TC ₆ , T ₂ C ₃), (C ₃)
Subgroup III			
4, N-tropic Balb/c	135	NIH 3T3	(C, C ₅ , TC), (T ₂ C, T ₂ C ₂), (TC ₆ , C ₂), (C ₃)
5, AKV-1	135	SC-1	
6, AKV-2	135	NIH 3T3	
7, AKV-2	135	SC-1	

The order of first appearance of the dC containing oligopyrimidines within the strong stop DNA sequence was determined by analysis of DNA molecules of discrete length synthesised *in vitro*. Reaction conditions were as described in the legend to Fig. 1 except that the concentration of [α -³²P]dCTP (100 Ci mmol⁻¹) was 10 μ M. After purification and alkaline hydrolysis the DNA products were layered on 20% polyacrylamide-urea gels. The DNA products were located by autoradiography, excised and eluted from the gels and the depurinated products were analysed as described elsewhere. The data for the Mo-MuLV virus was obtained from Mo-MuLV grown on NIH 3T3 cells and NRK cells. The length in nucleotides of the DNA products depurinated were 20, 30, 40, 60, 75 and 135. The N- and B-tropic BALB/c viruses are the cloned isolates described by Jolicouet *et al.*²³. The AKV-1 and AKV-2 virus were cloned isolates grown on SC-1 cells provided by J. Hartley. The length in nucleotides of the DNA products depurinated was 30, 45, 60 and 135 and for the N-tropic and AKV-1 and AKV-2 viruses; and 30, 45, 75 and 135 for the B-tropic BALB/c virus.

viruses and the feline viruses of the feline leukaemia (FeLV) class. The second group, strong stop DNA 120 $\pm$ 5, includes the endogenous baboon viruses and the endogenous feline virus RD114. The chicken viruses comprise the third group, strong stop DNA 101 $\pm$ 2. The strong stop DNA of the murine mammary tumour virus tested is clearly longer, 150 $\pm$ 5 nucleotides, than that of the other murine viruses.

The notion that viruses grouped together in Table 1 are more closely related to each other than they are to viruses in another group is supported by studies of sequence homology by nucleic acid hybridisation. For example, there is substantial sequence homology among the murine viruses¹⁰⁻¹³ and between some of the murine viruses and the woolly gibbon viruses^{8,13,14}. There is low, but detectable homology between the murine and feline leukaemia viruses^{10,13}. Nucleic acid hybridisation experiments reveal no similarity between the viruses of the different groups defined in Table 1.

The relationships amongst the viruses suggested by Table 1 are also supported by data on the immunological cross-reactivity of the virus specific proteins. The mammalian C-type viruses share broad interspecies determinants for the major internal structural protein, the reverse transcriptase, and the envelope glycoprotein, as well as for some of the lower molecular weight structural proteins, but do not share such interspecies determinants with either the chicken or the B-type murine viruses¹⁵⁻¹⁸. Antigenically the woolly-gibbon viruses are much more closely related to the murine viruses than they are to the baboon endogenous viruses or to RD114^{13,14,19}. The feline viruses are also immunologically related to the rodent viruses. The endogenous baboon viruses are closely related to one another and to the endogenous feline virus RD114^{17,20-22}.

Discrimination between closely related viruses by the oligopyrimidine composition of the strong stop DNA

Nucleotide sequence data regarding strong stop DNA provides additional information about the relationship amongst the viruses within the groups defined in Table 1. For this purpose AMV reverse transcriptase was used to transcribe purified viral 70S RNA in reactions in which the concentration of [α -³²P]dCTP was 10 μ M. At this low dNTP concentration, the reverse transcriptase has a high probability of terminating transcription at specific sites short of the 5' end of the genome, producing polynucleotide fragments shorter than strong stop DNA⁹. When the DNA products of such reactions are analysed on

polyacrylamide gels, a set of DNA products of discrete molecular weight is observed. The order of the appearance of the oligopyrimidine tracts within the strong stop DNA sequence can be deduced by depurination of a set of such DNA products. Such information has been presented for the chicken virus RSV PrC and for the murine virus Mo-MuLV⁹. The results of several such experiments are presented in Table 2.

The murine viruses

Many C-type viruses have been isolated from mice. These viruses are endogenous to this species, that is, proviral DNA sequences homologous to the replicating viruses are found in the DNA of all normal mice. The oligopyrimidine composition of the strong stop DNA of the murine viruses tested shows that the sequence of these viruses, at least near the 5' end of the genome, is very similar. However, some sequence differences are detected, and we have used these differences to define three subgroups of the murine viruses (Table 2). The sequence of Mo-MuLV, subgroup I, differs by a minimum of two pyrimidines from that of the ecotropic viruses of subgroup III. The estimated number of pyrimidine changes in the sequence is necessarily a minimum estimate because only oligopyrimidine tracts containing deoxycytidine were scored in this analysis.

Table 2 illustrates the order of the oligopyrimidines within the strong stop DNA sequence. The distinctive oligonucleotides C₃, C₅, TC₆ and T₂C₂ occupy similar positions within the sequence. In the case of Mo-MuLV, the sequence of the strong stop DNA confirms the locations of these oligonucleotides relative to the initiation point: C₅ 10-14; TC₆, 45-51; and C₃, 113-115 (A. Maxam, T. Hageman and W.H., unpublished observations).

The ecotropic murine viruses (those isolates capable of replication only on cell lines of murine origin) are subject to host range restriction controlled by the Fv-1 gene locus²³. Thus, N-tropic viruses grow only on cells of Fv-1ⁿ genotype, B-tropic virus on Fv-1^b cells, and NB-tropic virus grow on both cell types. The N-tropic, B-tropic and NB-tropic viruses tested fall into different subgroups (Table 2). Of the N-tropic viruses tested, N-tropic BALB/c virus and AKV-1 and AKV-2 all have the same oligopyrimidine composition. Moreover, the termination patterns of the N-tropic viruses are very similar (see Fig. 2) and are clearly distinct from those of the B- and NB-tropic viruses. There is evidence that the restriction by the Fv-1 locus operates to prevent some early step in virus replication, before integration of the provirus^{24,25}. The 5' sequence of the virus may be involved in several of the early

steps of RNA tumor virus replication; the synthesis of a complete strand of DNA complementary to the viral RNA through a jumping mechanism utilising the redundant sequence at the 5' end of the genome and integration at specific sites on the chromosome homologous to the redundant sequence^{1,26,27}.

The woolly-gibbon viruses

Viruses have been isolated from a woolly monkey with fibrosarcoma²⁸⁻³⁰, the brains of normal gibbons¹⁹, and the tissues of normal and leukaemic gibbons³¹⁻³³. Analyses of oligopyrimidine tracts show that the strong stop DNA sequences of this family of viruses are very similar to one another. Using pyrimidine tract data we have separated these viruses into the three subgroups shown in Table 3. A minimum of two pyrimidine changes in the sequence of the strong stop DNA could produce the changes in the two oligopyrimidines that differ between the woolly monkey virus (subgroup I) and the gibbon brain viruses (subgroup II). A minimum of three pyrimidine changes is required to account for the difference between subgroups I and III and two changes between subgroups I and II and between subgroups II and III. Table 3 shows that the larger oligopyrimidines, C₅, T₂C₂ and C₃, occupy similar positions within the strong stop DNA sequences. The oligopyrimidines T₂C₅, T₄C₂ of the gibbon leukosis virus occupy the same relative positions in the sequence as do the TC₆ and T₃C₂ of the woolly gibbon brain viruses and could be related by single nucleotide changes. Table 3 also shows that isolates of SSAV propagated on human, marmoset or rat cells have the same oligopyrimidine composition.

The oligopyrimidine tracts of the gibbon brain isolates, GBR-1 and GBR-2 are identical even though the viruses are isolated from different gibbons and propagated on different cell lines¹⁹. From these and other observations (see also Tables 3, 4 and 5) we conclude that the length and oligopyrimidine composition of strong stop DNA is independent of the cells on which the virus is propagated. The observation that only one strong stop pattern is observed for the mixture of transforming and helper viruses SiSV (SSAV) suggests that either the strong stop sequence of the defective SiSV genome is the same as that of the SSAV helper or that the defective genome accounts for only a small fraction of the DNA synthesised.

The subgroups of the woolly-gibbon viruses defined here are similar to those previously described¹. These woolly-gibbon

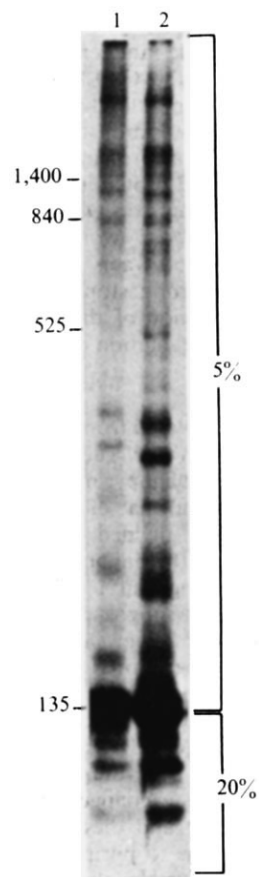


Fig. 2 Analysis of the DNA products of the N- and B-tropic BALB/c murine viruses. The salts and buffers of the reaction were as described in Table 1. The concentration of the limiting dNTP was 20 μ M [α -³²P]dCTP, the concentration of Nonidet was 0.01%, and the concentration of virions was 2 mg ml⁻¹. After purification and alkaline hydrolysis, the DNA products were layered on a composite 5%–20% polyacrylamide-urea gel. The size markers (not shown) were adenovirus DNA fragments of known length.

Table 3 The 5' to 3' order of the oligopyrimidines that contain dC of woolly-gibbon virus strong stop DNAs

Virus	Length of strong stop DNA	Cell	The 5' to 3' order of the oligopyrimidines that contain dC
Subgroup I			
Woolly helper			
1, SSAV-1	135	Human	(C, C ₅ , TC), (T ₂ C ₂ , C ₂ , TC ₆), (C ₃ , TC ₂ , T ₃ C ₃)
2, SSV (SSAV-1)	135	Human	
3, SSV (SSAV) 71AP1	135	Marmoset	
4, SSAV NC37	135	Human lymphoid	
5, SSAV (M55)	135	Rat	
6, SSAV	135	Human	
Subgroup II			
Gibbon brain			
7, GBR—1	135	Human	(C, C ₅ , TC), (T ₂ C, T ₂ C ₂ , C ₂ , TC ₆), (C ₃ , TC ₂ , T ₃ C ₂)
8, GBR—2	135	Human	
9, GBR—2	135	Bat lung	
Subgroup III			
Gibbon LV			
10, Gibbon S. F.	135	Human	(C, C ₅ , TC), (T ₂ C, T ₂ C ₂ , C ₂ , T ₂ C ₅), (C ₃ , TC ₂ , T ₄ C ₂)
11, Gibbon S. F.	135	Human	

The order of first appearance of the oligopyrimidines containing dC within the strong stop sequence was determined as described in the legend to Table 2. The SSAV-1 and SSV(SSAV-1) of lines 1 and 2 were grown on human A204 cells. The SiSV(SSAV) 71AP1 were grown on marmoset cells, the SSAV. NC37 were grown on the human lymphocyte line NC37; SSAV(M55) was grown on NRK (rat kidney cells), and the SSAV was grown on human A375 cells. This preparation was identified as SSAV by a type specific anti-p12 radioimmune precipitation assay (S. A. Aaronson, personal communication). The gibbon brain isolates are those previously described¹⁹. GBR-1 was grown on human A204 cells. GBR-2 was grown on human A204 and bat lung cells. The two preparations of the gibbon leukosis virus (San Francisco isolates) were grown on human A204 cells. For these experiments the lengths in nucleotides of the DNA products depurinated were 30, 75 and 135. Lines 1, 2 and 7–11 were obtained from G. Todaro's, laboratory; lines 2–5 from R. C. Gallo's laboratory and line 4 from S. A. Aaronson.

viruses contain immunologically related reverse transcriptases^{34,36} and group-specific antigens (p30 proteins)^{15,36-38}. The woolly-monkey virus, the GALV-San Francisco and the gibbon brain isolates can be clearly distinguished from one another by molecular hybridisation techniques¹⁹, by antigenic characterisation of the p12 core proteins^{39,40} and by the specificity of *in vitro* binding of the p12 protein to viral RNAs.

The sequence of the strong stop DNA of the murine viruses closely resembles that of the woolly-gibbon viruses. The characteristic oligopyrimidine tracts C₅, TC₆ and C₃ occupy similar positions in the strong stop DNA sequences of the viruses. Apparently this region of the virus genome is highly conserved in the course of evolution of these viruses⁴¹.

The baboon viruses

C-type viruses have been isolated from normal tissues of several different species of baboons⁴²⁻⁴⁶. Table 4 lists the length of the strong stop DNA and the oligopyrimidine tracts derived from the baboon virus species and that of the endogenous cat virus RD114. These viruses are divided into four subgroups on the basis of the length and the oligopyrimidine composition of the strong stop DNA: subgroup I (M-7 prototype) isolated from *Papio cynocephalus*⁴³; subgroup II (M-28⁴² and BILN⁴⁴ prototypes) isolated from *Papio cynocephalus/anubis* and *Papio hamadryas* respectively; subgroup III (PP-1 prototype) isolated from *Papio papio*⁴⁵; subgroup IV feline endogenous (RD114 prototype) isolated from feline tissues. The only differences between the subgroups I, II and III are in the longest oligopyrimidine tract located very near the 3' end of the sequence. A minimum of one pyrimide change separates the subgroup I and subgroup II viruses, and these are distinguished by a minimum of one pyrimidine to purine change from the *Papio cynocephalus* virus, M-7. Thus, the oligopyrimidine composition of the strong stop DNA, particularly the long 3' terminal oligopyrimidine tract, may serve as a marker for the species of the origin of these viruses. The oligopyrimidine tracts of the RD114 are markedly different from those of the baboon viruses.

The subgroups of the baboon viruses described here are similar to those defined by other techniques. Molecular hybridisation techniques detect differences between the baboon viruses and RD114. Antigenic differences between the p30 core proteins and the reverse transcriptases of the baboon viruses and RD114 have also been reported⁴⁷⁻⁴⁹.

The chicken viruses

We have studied some of the numerous C-type viruses isolated from chickens⁵⁰. The viruses studied include the Rous sarcoma viruses, Prague B (PrB) and Prague C (PrC), a non-transforming variant of PrB, the avian myeloblastosis virus (AMV), a Rous associated virus (RAV-1), the inducible endogenous chicken virus (RAV-0), and a recombinant between RAV-1 and an endogenous chicken provirus RAV-60 (RAV-1)⁵¹. The strong stop of these viruses is 101 ± 2 bases long. These viruses are divided into the four subgroups listed in Table 5. The relative molar yield of each oligopyrimidine is also shown.

The subgroup I viruses include RSV-PrB, RSV-PrC and their nontransforming variants. These are distinguished from AMV by T₂C. The molar yields of TC and T₂C distinguish RAV-0 from the subgroup I and subgroup II viruses. RAV-1 and the RAV-60 derived from RAV-1 have identical oligopyrimidine composition.

The subgroups of the chicken viruses defined in Table 5 provide a new method for classifying the chicken viruses. Other schemes depend on host range and transformation activity. Analysis of the strong stop DNA can reveal which parent contributes the 5' end of the genome to recombinant progeny. Thus, the 5' end of the RAV-60 recombinant studied here is probably derived from the RAV-1 parent.

Discussion of the technique

For each of the viruses analysed here, the shorter DNA products are incomplete transcription products of a longer sequence, and all the DNA products are initiated at a common site. This is deduced from the order of appearance of the long oligopyrimidine tracts.

Characterisation of virus by analysis of the oligopyrimidine tracts requires that the DNA molecules of discrete length comprise a substantial fraction of the reaction product. This was the case for the viruses analysed. If the template is not reasonably intact, or if the 70S RNA is not pure, then a high background of DNA molecules of all lengths is evident. Analysis of oligopyrimidines of DNA bands cut from gels with high background produces spurious tracts.

Depurination of DNA products of increasing length provides a method for checking the purity of a virus preparation. If a contaminating virus were present in substantial amounts, then

Table 4 The 5' to 3' order of the oligopyrimidines that contain dC of the baboon viruses and of RD114 strong stop DNAs

Virus	Length of strong stop DNA	Species of origin	Cell	5' to 3' order
Subgroup I				
1, Endogenous baboon virus M-7	120	<i>P. cynocephalus</i>	Human	(C, C ₂), (T ₄ C ₂),
2, Endogenous baboon virus M-7	120	<i>P. cynocephalus</i>	Canine thymus	(C ₃ , TC), (T ₃ C), (TC ₂ , T ₃ C ₇)
3, Endogenous baboon virus M-7	120	<i>P. cynocephalus</i>	Canine thymus	
4, Endogenous baboon virus M-7	120	<i>P. cynocephalus</i>	Canine thymus	
Subgroup II				
5, Endogenous baboon virus M-28	115	<i>P. cynocephalus/anubis</i>	Bat lung	(C, C ₂), (T ₄ C ₂),
6, Endogenous baboon virus 455K	115	<i>P. cynocephalus/anubis</i>	Canine thymus	(C ₃ , TC), (T ₃ C), (TC ₂ , TC ₆)
7, Endogenous baboon virus 455K	115	<i>P. cynocephalus/anubis</i>	Human A204	
8, Endogenous baboonvirus BILN	115	<i>P. hamadryas</i>	Cf ₂ Th	
Subgroup III				
9, Endogenous baboon virus PP-1	120	<i>P. papio</i>	Rhesus lung	(C, C ₂), (T ₄ C ₂),
10, Endogenous baboon virus PP-1	120	<i>P. papio</i>	Rhesus lung	(C ₃ , TC), (T ₃ C), (TC ₂ , T ₂ C ₅)
Subgroup IV				
11, RD114 (feline)	115	Feline	Human rhabdomyosarcoma	(C, C ₂ , TC, TC ₅),
12, RD114 (feline)	115	Feline	Human rhabdomyosarcoma	(TC ₃ , T ₂ C ₃)

The order of first appearance of the oligopyrimidines containing dC within the strong stop sequence was determined as described in Table 2. The endogenous baboon virus, M-7 was grown on human A204 cells or canine thymus cells, lanes 2, 3 and 4. The species of origin of M-7 is reported to be *Papio cynocephalus*⁴³. The M-28 virus⁴² was grown on bat lung cells. The 455K on human A204 and on canine thymus cells. The species of origin of these viruses is *Papio cynocephalus* (perhaps the anubis subspecies; R. Smith, personal communication). The BILN virus was grown on Cf₂Th cells and *Papio hamadryas* is the reported species of origin⁴⁴. Both preparations of PP-1 virus were grown on rhesus lung cells. This virus was isolated from *Papio papio*. The length in nucleotides of the DNA products depurinated were 30, 40, 60, 75, and 115. Both RD114 viruses were grown on human rhabdomyosarcoma cells. The length in nucleotides of the DNA products depurinated were 75 and 115. The virus preparations were supplied by the laboratories of G. Todaro (lines 1, 2, 9 and 10), R. Gallo (3, 6-8, 11 and 12) and R. Gilden(4).

Table 5 Quantitation and 5' to 3' order of the oligopyrimidines that contain dC of the chicken group

Virus Subgroup I	Length of strong stop DNA	Cell	5' to 3' order of the oligopyrimidines containing dC
1, RSV PrB	101	CEF	C, C ₂ (2), C ₃ (1), TC(3), TC ₂ (1), T ₂ C(2), T ₂ C ₂ (1), T ₃ C ₃ (1) (C, T ₃ C ₃), (T ₂ C ₂ , T ₂ C), (TC, TC ₂ , C ₂), (C ₃ , TC)
2, RSV PrB td			
3, RSV PrC			
Subgroup II			
4, AMV	101	CEF	C, C ₂ (2), C ₃ (1), TC(3), TC ₂ (1), T ₂ C ₂ (1), T ₃ C ₃ (1) (C, T ₃ C ₃), (T ₂ C ₂ , TC, TC ₂ , C ₂), (C ₃ , TC)
Subgroup III			
5, RAV-0	101	CEF	C, C ₂ (1), C ₃ (1), TC(4), TC ₂ (2), T ₂ C(1), T ₂ C ₂ (1), T ₃ C ₃ (1) (C, T ₃ C ₃), (T ₂ C ₂ , T ₂ C, TC, TC ₂ , C ₂), (C ₃ , TC)
Subgroup IV			
5, RAV-1	101	CEF	C, C ₂ (2), C ₃ (1), TC(4), TC ₂ (2), —, —, T ₂ C ₂ (1), T ₃ C ₃ (1) (C, T ₃ C ₃), (T ₂ C ₂ , TC, TC ₂ , C ₂), (C ₃ , TC)
6, RAV-60 (RAV-1)			

The order of first appearance of the oligopyrimidines that contain dC within the strong stop DNA sequence was obtained as described in Table 2. The relative molar yield was calculated by determining the amount of ³²P in each oligopyrimidine tract and then dividing by the specific activity and by the number of dC residues it contained. The chicken viruses were grown on chick embryo fibroblasts. The RSV PrB and PrB td strains were provided by J. Coffin. The nondefective or transformation defective property of the virus stocks were determined by transformation assays and fingerprint analysis. The nondefective strain used was > 90% pure. The RSV PrC and AMV (plasma virus) were supplied by the Office of Program Resources and Logistics. The RAV-0, RAV-1, and RAV-60 (RAV-1) virus preparations were supplied by G. Cooper. The length of the DNA products depurinated were: subgroups I and II—15, 30, 60 and 135; subgroups II and IV—15, 60, and 135.

some of the DNA chains of discrete length would produce oligopyrimidine tracts that would be inconsistent with a nested set of sequences.

The pattern of discrete DNA products synthesised at low dNTP concentration that can be visualised by autoradiography of the polyacrylamide gels serves as a rapid test for the similarity or difference of viral isolates. The pattern of banding is reproducible for viruses grown on different cell lines and even on cell lines of different species.

Sequence of strong stop DNA

The sequence of the strong stop DNAs of RSV PrC^{4,5}, AMV⁶ and Mo-MuLV (A. Maxam, T. Hageman and W.H. unpublished) have been determined. These sequences offer a very sensitive means for quantitative determination of the differences between the virus isolates. The strong stop DNAs of RSV and AMV are 101 bases long and differ in nine positions, four of which are pyrimidines. Two of these four pyrimidine differences are reflected in the oligopyrimidines that contain dC. The absence of the oligopyrimidine T₂C in the AMV sequence is accounted for by two differences in the AMV sequence, the T of RSV Pr-C at position 21 is a G in AMV, and the T of RSV Pr-C at position 28 is a C in AMV.

A limitation of the method described above is that the strong stop DNA comprises only about 1% of the viral genome. However, DNA transcripts can be synthesised *in vitro* that represent full-length copies of the viral RNA⁵²⁻⁵⁴. In the case of the Mo-MuLV these transcription products represent elongation of the strong stop DNA sequence by copying of the 3' end of the viral genome⁵⁵. Depurination of such elongated products provides information regarding the 3' sequence of the genomes⁹. Viruses may be compared over a substantial portion of the length by this technique. For example, Fig. 2 shows a side-by-side comparison of the DNA products up to 3,000 nucleotides long, synthesised from the 70S RNA of the N- and B-tropic BALB/c viruses. The bands in the upper and lower regions of the gel in adjacent tracts line up, but those in the region between 250 and 300 nucleotides do not. This pattern is consistent with a 50-nucleotide insertion/deletion about 200 bases from the point of DNA initiation.

The method for the analysis of retroviruses presented here can be used to detect similarities between distantly related viruses, that is, endogenous baboon virus and the feline virus RD114 as well as to discriminate between very closely related viruses, that is, *Papio papio* and *Papio cynocephalus* viruses.

Sequence analysis can detect a single base difference between two virus isolates. The virus categories defined by this method are consistent with existing data on the relationship of the virus studied. The method described here complements existing classification techniques. In addition, this scheme provides detailed information about the 5' end of the genome, a sequence that may code for such diverse functions as initiation of translation, integration, subunit-subunit linkage, initiation of DNA synthesis, a circularisation of the genome, and post-transcriptional modification of the viral mRNA.

We thank S. Aaronson, J. Coffin, G. Cooper, R. Gallo, J. Hartley, N. Hopkins, M. Reitz, A. Sen, R. Smith and G. Todaro for gifts of viruses. We also acknowledge the support of the Massachusetts Institute of Technology Cell Culture Facility and the US NCI Office of Program Resources and Logistics. This work was supported by grants from the American Cancer Society, NCI (CA-19341), the Ruth Estrin Goldberg Memorial for Cancer Research, and the NIH general research support grant to Stanford Research Institute, (SRI RR-05522-13).

Received 21 October 1977; accepted 10 March, 1978.

1. Todaro, G. J., Benveniste, R. E., Sherr, C. J., Lieber, M. M. & Callahan, R. *Bibliotheca Haematologica* No. 43 (ed. Clemmens, J.) (D. S. Yohn, Copenhagen, 1976).
2. Taylor, J. M. & Illmensee, R. *J. Virol.* **16**, 553-558 (1975).
3. Collet, M. S. & Faras, A. J. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1329-1332 (1976).
4. Haseltine, W., Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 989-993 (1977).
5. Shine, J., Czernilofsky, A. P., Friedrich, R., Bishop, J. M. & Goodman, H. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1473-1477 (1977).
6. Stoll, E., Billeter, M. A., Palmenberg, A., & Weissmann, D. *Cell* (in the press).
7. Coffin, J., Haseltine, W. & Maxam, A. *Cell* (in the press).
8. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).
9. Haseltine, W. A., Kleid, D., Panet, A., Rothenberg, E. & Baltimore, D. *J. molec. Biol.* **106**, 109-121 (1976).
10. Benveniste, R. E., Todaro, G. J. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3316-3320 (1973).
11. Callahan, R., Benveniste, R. E., Lieber, M. M. & Todaro, G. J. *J. Virol.* **14**, 1394-1403 (1974).
12. Wong-Staal, F., Gallo, R. C. & Gillespie, P. *Nature* **256**, 670-679 (1975).
13. Benveniste, R. E., Callahan, R., Sherr, C. J., Chapman, V. & Todaro, G. T. *J. Virol.* **21**, 849-862 (1977).
14. Lieber, M. M. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **72**, 2315-2319 (1975).
15. Gilden, R. V. in *Viruses, Evolution and Cancer, Basic Considerations* (eds Kurstak, G. & Marmorosch, M.) (Academic, New York, 1974).
16. Hino, S., Stephanson, J. R. & Aaronson, S. A. *J. Virol.* **18**, 933-941 (1976).
17. Barbacid, M., Stephanson, J. R. & Aaronson, S. A. *Cell* **10**, 641-648 (1977).
18. Krakower, J. M., Barbacid, M., & Aaronson, S. A. *J. Virol.* **22**, 331-339 (1977).
19. Todaro, G. T. *et al.* *Virology* **67**, 335-343 (1975).
20. Hellman, A. *et al.* *J. Virol.* **14**, 133-138 (1974).
21. Donehower, L., Wong-Staal, F. & Gillespie, D. *J. Virol.* **21**, 932-941 (1977).
22. Sherr, C. J. & Todaro, G. J. *Science* **187**, 855-857 (1974).
23. Lilly, F. & Pincus, T. *Adv. Cancer Res.* **17**, 231-277 (1973).
24. Jolicouer, P. & Baltimore, D. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2236-2240 (1976).
25. Sveda, M. M. & Soeiro, R. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2356-2360 (1976).
26. Haseltine, W. A. & Baltimore, D. in *Animal Virology* (eds Baltimore, D., Huang, & Fox) **175-213** (Academic, New York, 1976).
27. Junghans, K. P., Hu, S., Knight, C. A. & Davidson, N. *Proc. natn. Acad. Sci. U.S.A.* **74**, 477-481 (1977).

28. Theilen, G. H., Gould, D., Fowler, M. & Dungworth, D. L. *J. natn. Cancer Inst.* **47**, 881-889 (1971).
29. Wolfe, L. G. *et al. J. natn. Cancer Inst.* **47**, 1115-1120 (1971).
30. Wolfe, L. G., Smith, R. K. & Deinhardt, F. *J. natn. Cancer Inst.* **48**, 1905-1908 (1972).
31. Kawakami, T. G. *et al. Nature new Biol.* **235**, 170-171 (1972).
32. Kawakami, T. G., Buckley, P. M., McDowell, T. S. & De Paoli, A. *Nature new Biol.* **235**, 842-843 (1973).
33. Kawakami, T. G. & Buckley, P. M. *Transplant. Proc.* **6**, 193-196 (1974).
34. Scolnick, E. M., Parks, W. P. & Todaro, G. J. *Science* **177**, 1119-1121 (1972).
35. Todaro, G. J. & Gallo, R. C. *Nature* **244**, 206-209 (1973).
36. Parks, W. P., Scolnick, E. M., Noon, M. C., Watson, C. J. & Kawakami, T. G. *Int. J. Cancer* **12**, 129-137 (1973).
37. Hoekstra, J. & Dienhardt, F. *Intervirology* **2**, 222-230 (1973).
38. Tronick, S. P., Stephanson, J. R., Aaronson, S. A. & Kawakami, T. G. *J. Virol.* **15**, 330-342 (1975).
39. Tronick, S. R., Stephanson, J. R., Aaronson, S. A. & Kawakami, T. J. *J. Virol.* **15**, 115-120 (1975).
40. Tronick, S. R., Stephanson, J. R. & Aaronson, S. A. *Virology* **57**, 347-356 (1974).
41. Sen, A. & Todaro, G. T. *Science* **193**, 326-327 (1976).
42. Melnick, T. L. *Intervirology* **1**, 386-398 (1973).
43. Benveniste, R. E. *et al. Nature* **248**, 17-20 (1974).

44. Goldberg, R. J., Scolnick, E. M., Parks, W. P., Yakovlova, L. P. & Lapin, B. A. *Int. J. Cancer* **14**, 722-730 (1974).
45. Benveniste, R. E. & Todaro, G. T. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4513-4518 (1974).
46. Benveniste, R. E. & Todaro, G. J. *Nature* **252**, 456-459 (1974).
47. Sherr, C. J., Lieber, M. M., Benveniste, R. E. & Todaro, G. J. *Virology* **58**, 492-503 (1974).
48. Sherr, C. J. & Todaro, G. J. *Virology* **61**, 168-181 (1974).
49. Benveniste, R. E., Sherr, C. J., Lieber, M. M., Callahan, R. & Todaro, G. T. in *Fundamental Aspects of Neoplasia* (eds Gottlieb, A., Plescia, O. & Bishop, D. J. L.), 29-53 (Springer, New York, 1975).
50. Tooze, J. in *The Molecular Biology of RNA Tumor Viruses* (ed. Tooze, J.) 539-540 (Cold Spring Harbor Laboratory, New York, 1973).
51. Hanafusa, T., Hanafusa, H. & Miyamoto, T. *Proc. natn. Acad. Sci. U.S.A.* **67**, 1797-1803 (1970).
52. Rothenberg, E. & Baltimore, D. J. *J. Virol.* **21**, 168-178 (1977).
53. Rothenberg, E. & Baltimore, D. J. *J. Virol.* **17**, 168-174 (1976).
54. Junghans, R. P., Duesberg, P. H. & Knight, C. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4895-4899 (1975).
55. Haseltine, W., Coffin, J. & Hageman, T. *Cell* (in the press).
56. Rickard, C. G., Barr, L. M., de Noronha, F., Dougherty, E. & Post, J. E. *Cornell Vet* **57**, 302-307 (1967).
57. Snyder, S. P. & Theilen, G. H. *Nature* **221**, 1074-1075 (1969).
58. Gardner, M. B. *et al. Nature* **226**, 807-809 (1970).

letters to nature

Positions of galactic

X-ray sources: $55^\circ < l^{\text{II}} < 320^\circ$

PRECISE (20-40") celestial positions of 10 galactic X-ray sources in the region $55^\circ < l^{\text{II}} < 320^\circ$ are reported here. These positions were measured with the rotating modulation collimator detectors on the SAS-3 X-ray observatory. The data were taken as part of a systematic survey of the galactic plane¹⁻⁷. Five positions reaffirm the identification of optical counterparts for 4U0614+09 (refs 8, 9), 2S0053+604 (refs 1, 10), 4U1145-61 (refs 1, 11-13), GX301-2 (refs 1, 12, 14), and GX304-1 (refs 1, 15). Positions for the latter four sources and for 4U0142

+61 are refinements of previously reported SAS-3 positions^{1,16}. The position for 2S0114+650 has led to the discovery of an optical counterpart^{17,18}. Positions for 4U0142+61, 4U0919-54, A1239-59, and 4U1254-69 are also presented. The optical counterparts of these sources are still to be discovered. The source 2S0114+650 was discovered as part of this work.

The positions reported here are derived from two independent observations with the exceptions of 2S0053+604 and 2S0114+650 (one observation each) and 2S0142+614 (three observations). Most observations yielded independent measurements from the 2.3' FWHM detector and the 4.5' FWHM detector. The observations (1-2 d each with typical effective exposure

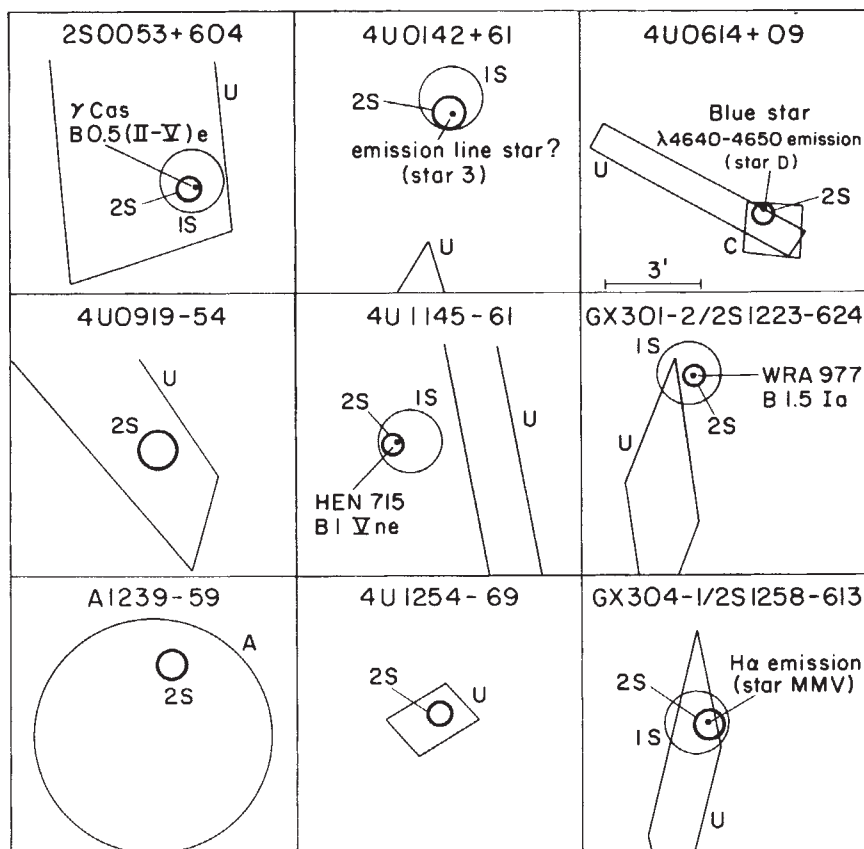


Fig. 1 SAS-3 error circles for X-ray sources compared with other measurements. The source 2S0114+650 is not displayed as no other measurements are available. The positions are from the 4U catalogue (U, ref. 32), the Copernicus satellite (C, ref. 33), the Ariel V rotating modulation collimator (A, ref. 45), and SAS-3. The larger SAS-3 circles (IS) are from refs 1 and 16; the smaller dark circles (2S) are from the present work. The scale for all sources is the same as that given for 4U0614+09. North is up and east is to the left. (References to the optical objects are given in the text.)

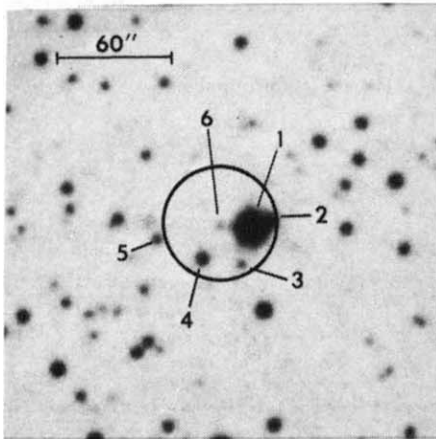
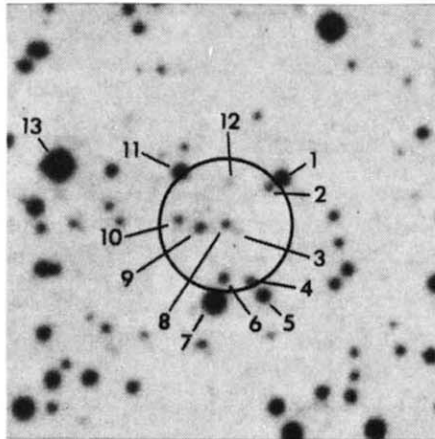
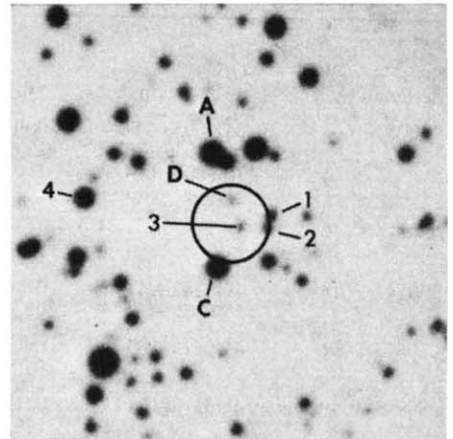
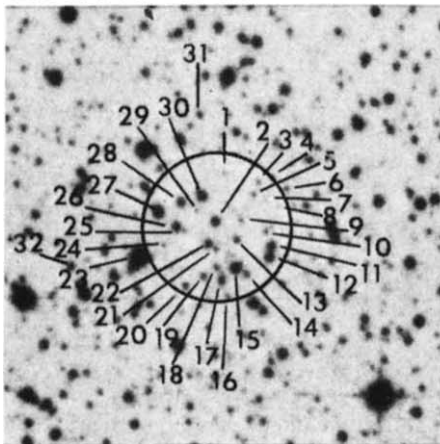
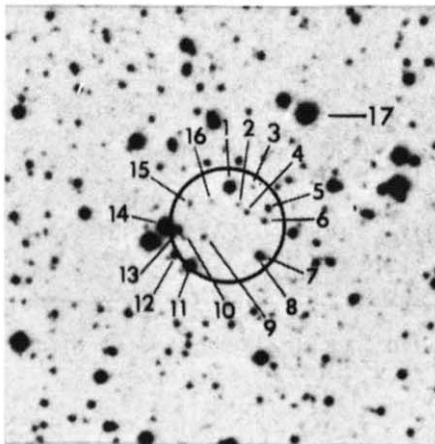
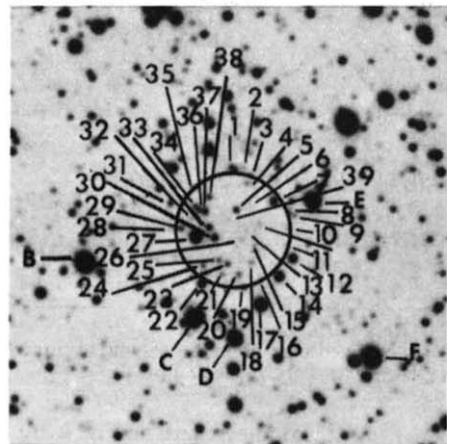
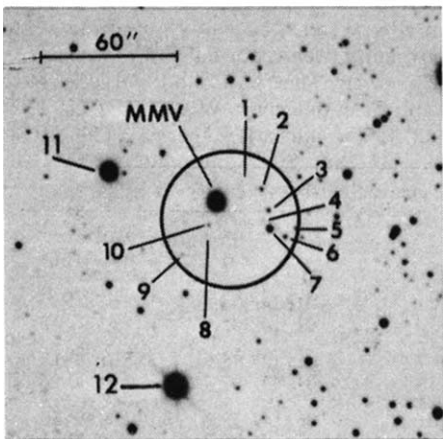
2S0114+650**2S0142+614****2S0614+091****2S0918-549****2S1239-599****2S1254-690****2S1258-613**

Fig. 2 Finding charts for the SAS-3 positions. The charts are from the Palomar Observatory Sky Survey (POSS) (at National Geographic Society) E (red) prints for 2S0114+650 and 2S0142+614, POSS O (blue) print for 2S0614+091, European Southern Observatory (ESO) quick-blue survey for 2S0918-549 and 2S1239-599, and B and R plates, respectively, taken with the CTIO 4 m telescope by C. R. Canizares and W. A. Hiltner for 2S1254-690 and 2S1258-613. The scales for the POSS and ESO charts are the same as that indicated for TS0114+650. The scales for the 4 m charts are the same as that indicated for 2S1258-613. North is up and east is to the left. Letter designations are from previous finding charts^{1,8,51}.

of 2.5×10^4 s) were carried out from June 1975 to November 1977. A deep exposure (2×10^5 s) in November 1977 yielded position measurements for 4U0142+61 (included here) and for the low-latitude extragalactic source 4U0241+61 reported elsewhere¹⁹. The detector system experienced low temperatures during some of the observations. Temperature-dependent calibration parameters⁷ were used to analyse these data. In all cases positions measurements obtained from observations made at low temperatures are consistent with position measurements obtained from other observations made near the normal

operating temperature ($\sim 0^\circ$ C) of the experiment.

Positions, error radii, and intensities (2–11 keV) of the 10 sources are given in Table 1. In addition we obtained positions (20" error radii) for 4U0900-40, Cen X-3, and Cyg X-3 that lie 10–15" from the positions of the optical²⁰⁻²² and radio-infrared^{23,24} counterparts. This supports the validity of our calibration procedures based on data from Sco X-1, Cyg X-1, and Cyg X-2 (ref. 2). The SAS-3 error circles are compared to other position determinations in Fig. 1. Finding charts for seven of the sources are given in Fig. 2. Precise stellar positions

Table 1 Celestial positions

SAS-3 designation	Other designations*	Position (1950)		l^{II} b^{II}	Error radius (90%)	Flux density† (2–11 keV)	Comments
		α	δ				
2S0053+604	MX0053+60 MX0049+59(?) 1S0053+604 4U0054+60	00h 53m 42.3s 13.4262°	60° 26' 43'' 60.4452°	123.6° –2.1°	25''	8 μ Jy	γ Cas ^{1,10, 25–29}
2S0114+650		01 14 44.3 18.6846	65 01 34 65.0261	125.7 2.6	30	4	Optical counterpart ^{17,18} (Star 1)
2S0142+614	4U0142+61 1S0142+615	01 42 55.1 25.7296	61 29 59 61.4997	129.4 –0.4	35	4	Possible counterpart ¹⁸ (Star 3)
2S0614+091	4U0614+09	06 14 22.4 93.5935	09 09 11 9.1531	200.9 –3.4	20	32	Optical counterpart ^{8,9} (Star D)
2S0918–549	4U0919–54	09 18 55.6 139.7317	–54 59 33 –54.9925	275.9 –3.8	40	10	
2S1145–619	4U1145–61 1S1145–619	11 45 35.3 176.3971	–61 55 49 –61.9302	295.6 –0.2	20	68	Optical counterpart ^{11,11–13} (HEN 715 – SAO251595) 297 s X-ray period ³⁵
2S1223–624	GX301–2 4U1223–62 1S1223–624	12 23 48.8 185.9535	–62 29 37 –62.4938	300.1 –0.0	20	42 (June 1975) 17 (April 1976)	Optical counterpart ^{11,12,14} (WRA 977) 23d optical period ^{40,41} ~699 s X-ray ⁴⁴ ~690 s optical period(?) ^{42,43}
2S1239–599	A1239–59	12 39 07.5 189.7811	–59 55 39 –59.9275	301.8 2.6	30	10	191 s X-ray period(?) ⁴⁶
2S1254–690	4U1254–69	12 254 19.7 193.5820	–69 01 03 –69.0174	303.5 –6.4	25	29	
2S1258–613	GX304–1 4U1258–61 1S1258–613	12 58 11.0 194.5458	–61 20 06 –61.3349	304.1 1.2	30	7	Optical counterpart ¹⁵ (Star MMV) 272 s X-ray period ^{46,49}

*Refs 1, 10, 32, 37, 45, 50.

†Averaged over observations and accurate to ~10%. 1.0 μ Jy corresponds to 2.2×10^{-11} erg cm⁻² s⁻¹ (2–11 keV). $l_{\text{crab}} = 1,060 \mu\text{Jy}$ (see refs 1, 2).

(≤3'') for the finding charts in Fig. 2 are given in Table 2.

We summarise below the status of each source with regard to its distinguishing X-ray properties and any proposed optical counterparts.

2S0053+604: This source was discovered during the galactic plane survey and identified with γ Cas^{1,10}. The X-ray source was discovered independently with the Ariel 5 satellite²⁵. γ Cas is a highly variable Be star which has been discussed elsewhere^{26–29}.

2S0114+650: This source, discovered in the present survey, has been associated by Margon *et al.*^{17,18} with a $V \sim 11$ star (star 1 on the finding chart) that shows broad H α emission and HeI absorption. The star is classified as an OB star in earlier catalogues^{30,31}. This source is distinct from the well-located (0.0039 deg²) transient source 4U0115+63 (ref. 32) that lies 1.5° to the south.

4U0142+61: A possible optical counterpart of this source (star 3 on Fig. 2) has been noted by Margon *et al.*¹⁸ on the basis of the present SAS-3 position. It is a faint star with a weak emission feature.

4U0614+09: This source is highly variable over long time periods (variability factor of 5 in the Uhuru catalogue³²). The flux density observed in the present work is a factor of four less than that listed in the Uhuru catalogue. On the basis of a Copernicus satellite position³³, an optical counterpart (star D on the finding chart) has been proposed by Murdin *et al.*⁸ who noted the longterm variability of the star and the similarity of its colours to those of Sco X-1. Davidsen *et al.*⁹ failed to find any large variability in the X-ray source or in the star during a few days of observations. However, they confirmed the similarity to the colours of Sco X-1 and noted the $\lambda 4,640$ – $4,650$ emission feature in the spectrum of the star. They estimated the distance of star D as 4–8 kpc and noted that the X-ray flux scales properly for a source similar to Sco X-1.

4U0919–54: No optical candidate has been suggested for this source.

4U1145–61: We have established¹ the identification of the previously suggested^{11–13} optical counterpart, Hen 715 – SAO251595 = HD102567, on the basis of an early SAS-3 position. This counterpart is a $V = 8.95$ (ref. 12) star with spectral classification B1Vne (ref. 34). X-ray pulsations with a period of 297 s have recently been reported³⁵.

GX301–2 (2S1223–624): This hard and variable X-ray source^{36–39} and its optical counterpart WRA 977 (refs 1, 12, 14) are discussed in ref. 1. The star, a Be supergiant ($V = 10.84$)

Table 2 Stellar Astrometry

Source	Star	Position (1950)*	
		α	δ
2S0114+650	1	01h14m41.8s	65°01'32''
	4	01 14 45.9	65 01 15
2S0142+614	7	01 42 55.5	61 29 18
	13	01 43 07.4	61 30 26
2S0614+091	C	06 14 22.7	09 08 50
	3	06 14 22.2	09 09 11
	4	06 14 27.5	09 09 24
2S0918-549	31	09 18 56.9	–54 58 32
	32	09 19 04.7	–54 59 55
2S1239-599	1	12 39 07.3	–59 55 17
	17	12 39 02.1	–59 54 37
2S1254-690	B	12 54 31.4	–69 01 17
	D	12 54 19.4	–69 01 51
2S1258-613	MMV	12 58 11.7	–61 19 57
	11	12 58 18.3	–61 19 43
	12	12 58 14.9	–61 21 18

*Accurate to $\lesssim 3''$.

first proposed by Vidal¹⁴, exhibits a 23 ± 1 d intensity variation^{40,41}. There have been reports^{42,43} of weak optical modulations from WRA977 with periods near the 699 s X-ray period⁴⁴. The two X-ray observations reported here show variability of the source by a factor of 2.5.

A1239-59: We detected this source with a flux density consistent with the flux observed by the Ariel V satellite⁴⁵. The source spectrum is quite hard and similar to that of GX304-1 (ref. 46). A1239-59 may be responsible for the 191 s pulsations detected by Ariel V in a wide field of view⁴⁶.

4U1254-69: No optical candidates have been suggested for this source.

GX304-1 (2S1258-613): This hard and variable X-ray source^{37-39,47,48} and its stellar counterpart (star MMV on Fig. 2) are discussed in ref. 1. The early-type star, proposed by Mason *et al.*¹⁵, exhibits broad H α emission. The X-ray flux is modulated with a pulse period of 272 s^{46,49}.

We thank the staffs of the Center for Space Research at MIT and the Goddard Space Flight Centre for their support, and also R. Kelley, E. Ng, and C. Reid for their assistance. We thank M. Liller, R. McCrosky, and C. Shao of the Harvard College Observatory for facilities for measuring of stellar positions. This work was supported in part by US NASA (contract NAS5-11450). This is the eighth and final article in a series on positions of galactic X-ray sources obtained with SAS-3.

R. G. DOWER
K. M. V. APPARAO*
H. V. BRADT
R. E. DOXSEY
J. G. JERNIGAN
J. KULIK

Department of Physics and Center for Space Research,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139

Received 6 February; accepted 8 March 1978.

*Permanent address: Tata Institute of Fundamental Research, Bombay, India.

1. Bradt, H. V. *et al.* *Nature* **269**, 21-25 (1977).
2. Doxsey, R. E., Apparaio, K. M. V., Bradt, H. V., Dower, R. G. & Jernigan, J. G. *Nature* **269**, 112-116 (1977).
3. Bradt, H. V. *et al.* *Nature* **269**, 496-497 (1977).
4. Jernigan, J. G., Apparaio, K. M. V., Bradt, H. V., Doxsey, R. E. & McClintock, J. E. *Nature* **270**, 321-323 (1977).
5. Doxsey, R. E., Apparaio, K. M. V., Bradt, H. V., Dower, R. G. & Jernigan, J. G. *Nature* **270**, 586-588 (1977).
6. Apparaio, K. M. V. *et al.* *Nature* **271**, 225-228 (1978).
7. Jernigan, J. G. *et al.* *Nature* **272**, 701-704 (1978).
8. Murdin, P. *et al.* *Mon. Not. R. astr. Soc.* **169**, 25-34 (1974).
9. Davidsen, A. *et al.* *Astrophys. J. Lett.* **193**, L25-L29 (1974).
10. Jernigan, J. G. *IAU Circ. No.* 2900 (1976).
11. Sofia, S. *Astrophys. J. Lett.* **188**, L45-L46 (1974).
12. Jones, C. A., Chetin, T. & Liller, W. *Astrophys. J. Lett.* **190**, L1-L3 (1974).
13. Maraschi, L., Treves, A. & van den Heuvel, E. P. J. *Nature* **259**, 292-293 (1976).
14. Vidal, N. V. *Astrophys. J. Lett.* **186**, L81-L83 (1973).
15. Mason, K. O., Murdin, P. G. & Visvanathan, N. *IAU Circ. No.* 3054 (1977).
16. Bradt, H., Doxsey, R., Jernigan, J. G. & Spada, G. *Bull. Am. Phys. Soc.* **21**, 544 (1976).
17. Margon, B. & Bradt, H. *IAU Circ. No.* 3144 (1977).
18. Margon, B., Kwitter, K., Bradt, H. V. & Dower, R. G. *Astrophys. J. Lett.* (to be submitted).
19. Apparaio, K. *et al.* *IAU Circ. No.* 3150 (1977); *Nature* (in the press).
20. Brucato, R. J. & Kristian, J. *Astrophys. J. Lett.* **173**, L105-L107 (1972).
21. Hiltner, W. A., Werner, J. & Osmer, P. *Astrophys. J. Lett.* **175**, L19-L22 (1972).
22. Krzeminski, W. *Astrophys. J. Lett.* **192**, L135-L138 (1974).
23. Braes, L. L. E. & Miley, G. K. *Nature* **237**, 506 (1972).
24. Becklin, E. E. *et al.* *Nature* **245**, 302-304 (1973).
25. Mason, K. O., White, N. E. & Sanford, P. W. *Nature* **260**, 690-691 (1976).
26. Hutchings, J. B. *Mon. Not. R. astr. Soc.* **150**, 55-66 (1970).
27. Moffat, A. F. J., Haupt, W. & Schmidt-Kaler, Th. *Astr. Astrophys.* **23**, 433-439 (1973).
28. Cowley, A. P., Rogers, L. & Hutchings, J. B. *Pub. astr. Soc. Pac.* **88**, 911-916 (1976).
29. Marlborough, J. M. *Pub. astr. Soc. Pac.* **89**, 122-126 (1977).
30. Gonzalez, G. & Gonzalez, G. *Bol. Obs. Ton. Tac.* **9**, 21 (1954).
31. Hardorp, J., Rohlfs, K., Slettebak, A. & Stock, J. *Luminous Stars in the Northern Milky Way*, vol. 1 (Warner and Swasey Observatory, 1959).
32. Forman, W. *et al.* *Astrophys. J. Suppl.* (in the press).
33. Willmore, A. P. *et al.* *Mon. Not. R. astr. Soc.* **169**, 7-23 (1974).
34. Feast, M. M., Stoy, R. H., Thackeray, A. D. & Wesselink, A. J. *Mon. Not. R. astr. Soc.* **122**, 239-253 (1961).
35. White, N. E. *IAU Circ. No.* 3118 (1977).
36. Lewin, W. H. G., McClintock, J. E., Ryckman, S. G. & Smith, W. B. *Astrophys. J. Lett.* **166**, L69-L72 (1971).
37. McClintock, J. E., Ricker, G. R. & Lewin, W. H. G. *Astrophys. J. Lett.* **166**, L73-L76 (1971).
38. Ricker, G. R., McClintock, J. E., Gerassimenko, M. & Lewin, W. H. G. *Astrophys. J.* **184**, 237-243 (1973).
39. Jones, C. *Astrophys. J.* **214**, 856-873 (1977).
40. Hammerschlag, Hensberge, G., Zuiderwijk, E. J. & van den Heuvel, E. P. J. *Astr. Astrophys.* **49**, 321-323 (1976).
41. van Genderen, A. M. *Astr. Astrophys.* **54**, 733-735 (1977).
42. Mauder, H. *IAU Circ. No.* 2946 (1976).
43. Chevalier, C., Motch, C. & Ilovaisky, S. A. *IAU Circ. Nos* 3146 and 3152 (1977).

44. White, N. E., Mason, K. O., Huckle, H. E., Charles, P. A. & Sanford, P. W. *Astrophys. J. Lett.* **209**, L119-L124 (1976).
45. Carpenter, G. F., Eyles, C. J., Skinner, G. K., Wilson, A. M. & Willmore, A. P. *Mon. Not. R. astr. Soc.* **179**, 27p-34p (1977).
46. Huckle, H. E. *et al.* *Mon. Not. R. astr. Soc.* **180**, 21p-26p (1977).
47. Lewin, W. H. G., Clark, G. W. & Smith, W. B. *Astrophys. J. Lett.* **152**, L49-L53 (1968).
48. Lewin, W. H. G., Clark, G. W. & Smith, W. B. *Nature* **219**, 1235-1236 (1968).
49. McClintock, J. E., Rappaport, S. A., Nugent, J. J. & Li, F. K. *Astrophys. J. Lett.* **216**, L15-L18 (1977).
50. Markert, T. H. *et al.* *Astrophys. J.* **218**, 801-814 (1977).
51. Penston, M. V., Penston, M. J., Murdin, P. & Martin, W. L. *Mon. Not. R. astr. Soc.* **172**, 313-324 (1975).

Discovery of 3.6-s X-ray pulsations from 4U0115+63

OBSERVATIONS of the recurrence of a previously known transient X-ray source 4U0115+63 (ref. 1) using SAS 3, and our discovery that it pulsates with a period of 3.61 s (ref. 2) are reported here. We also give a measurement of the source position³ which is precise to ~ 30 arc s and which has led directly to the identification of a likely optical counterpart^{4,5}. A preliminary determination of the orbital elements of the binary system has been made from measurements of the Doppler variations of the pulse period and has yielded an orbital period of ~ 24 d (ref. 5).

Only two transient X-ray sources were previously known to exhibit regular X-ray pulsations; A1118-61 (ref. 6) and A0535+26 (refs 7, 8). Both sources have hard X-ray spectra⁹ and are tentatively identified with Be stars^{10,11}. Although virtually nothing is known about the binary orbit of A1118-61, in the case of A0535+26, the available observations indicate that the orbital period is large (≥ 20 d)¹². However, the long pulse period of A0535+26 (104 s) has made precise Doppler measurements of the binary orbit extremely difficult. The discovery of short period X-ray pulsations from 4U0115+63, therefore, has presented a unique opportunity to advance our understanding of transient X-ray sources.

The new X-ray outburst of 4U0115+63 was observed with the Y-axis detectors of SAS 3 on 5.9 January 1978 (UT). At that time the intensity was ~ 0.17 times that of the Crab Nebula (1-27 keV)². Increased emission from this region of the sky was also detected independently by the Ariel 5 all sky monitor in data recorded on 30 December 1977 (UT)¹³. The source was scanned by SAS 3 once per satellite orbital period (~ 94 min) with both the centre and right slat collimator detectors^{14,15} (CSL and RSL) during an observation of another region of the sky by the rotation modulation collimator detectors (RMC)¹⁶. 4U0115+63 was observed in this mode until 10.5 January 1978 (UT) when the rotation of the satellite was stopped and the CSL was pointed at the source. Data were obtained from two orbits with 0.415 s time resolution (1-13 keV) and were then subjected to Fourier analysis. Figure 1 shows the resulting power density spectrum. A strong peak is seen at a frequency of ~ 0.28 Hz. The presence of strong first and second harmonics indicates that the pulse profile is sharply peaked. The pulse fraction is approximately 30% (1-13 keV) and increases with increasing energy.

The source was again scanned once per orbit with the Y-axis detectors from 10.8 to 12.6 January 1978 (UT). This was followed by a second pointed observation with the CSL during the interval 12.7-13.5 January 1978 (UT). Figure 2 is an observation log which indicates the times when 4U0115+63 was in the field of view of one of the detectors. The intensity of the source was always between 0.15 and 0.2 Crab (1-27 keV), and there is no evidence of any eclipse. The longest interval between successive sightings was ~ 0.35 d out of ~ 7.6 d total coverage. This represents the upper limit to the duration of any X-ray eclipse in the ~ 7.6 -d observation. For most of the observing period, however, the upper limit to the duration of an eclipse is ~ 0.065 d (~ 1.5 h) which was the time between successive scans over the source.

Crude spectral information was obtained for 4U0115+63

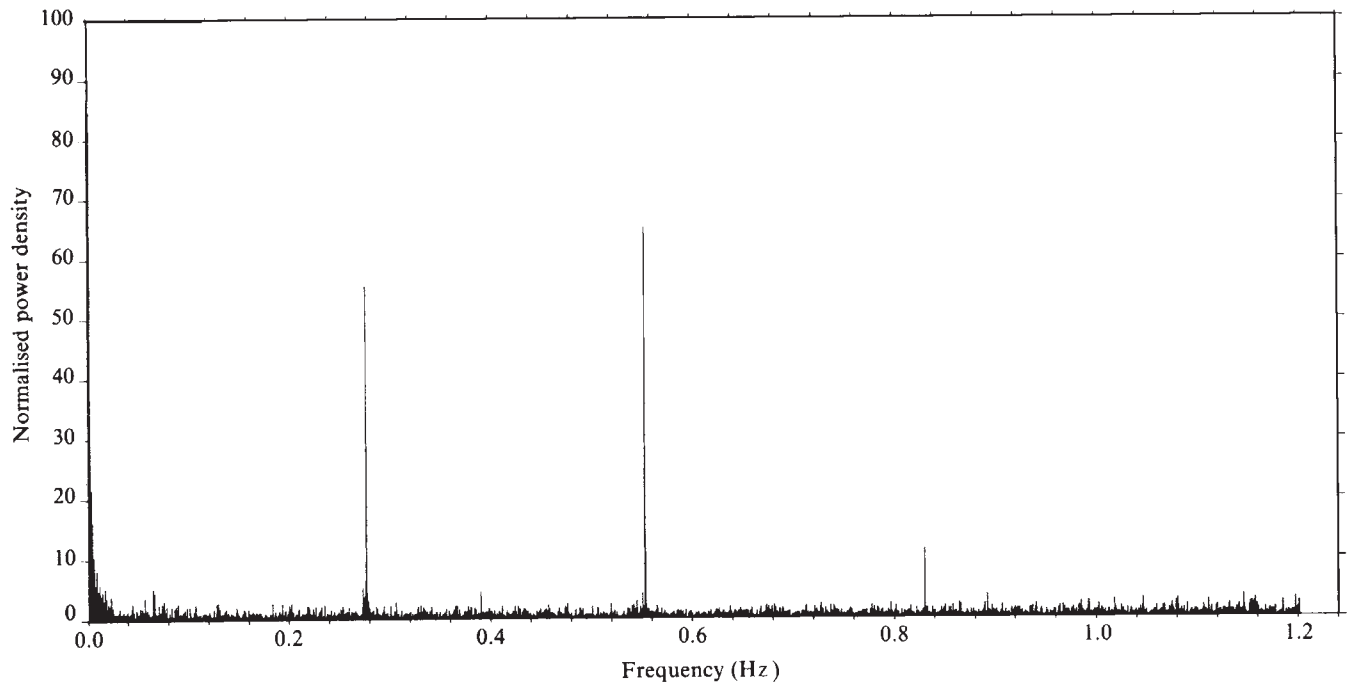


Fig. 1 Power density spectrum for 4U0115+63. The data were obtained with the SAS 3 Y-axis detectors during one satellite orbit near 10.5 January 1978 (UT).

from the ratios of counting rates in the first three energy channels of the CSL (1–27 keV). The counting rate decreases by less than a factor of three from the first (1–5 keV) to the third (13–27 keV) energy channels, and is detected at the 3.3σ level in the latter channel. The counting rate of the Crab Nebula in the same detectors decreases by a factor of five over this energy range. The spectrum of 4U0115+63 is, therefore, flatter than that of the Crab Nebula, consistent with results obtained from Uhuru¹⁷ and HEAO-1 (ref. 18) observations.

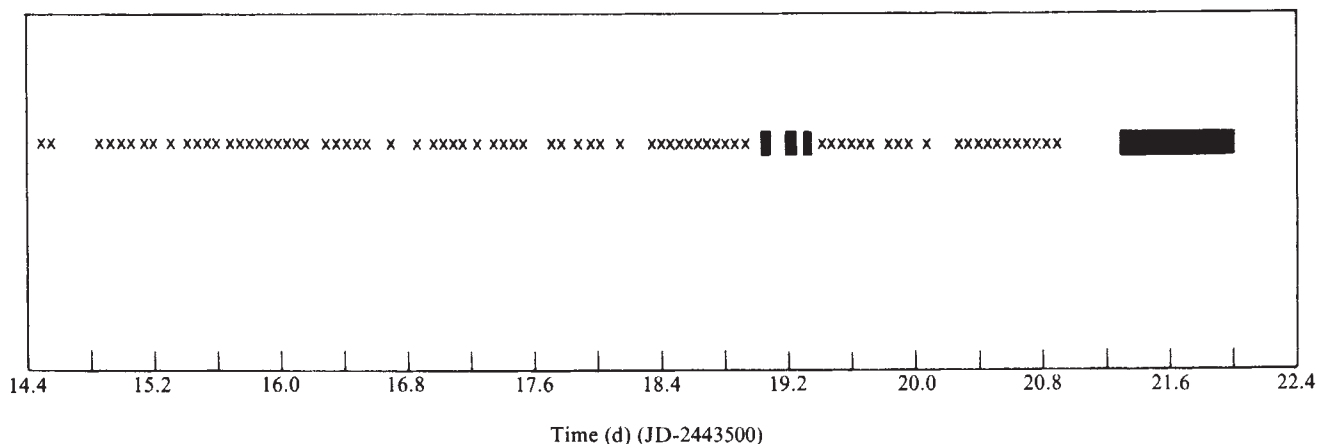
The observatory was next reorientated so that the position of 4U0115+63 could be determined with the RMC¹⁶. These observations were carried out between 15.1 and 16.7 January 1978 (UT). We used data obtained with both the 2.3 arc min and 4.5 arc min FWHM RMC detectors in each of two different satellite orientations. The four independent position determinations have an r.m.s. scatter of 10 arc s. The centre of the 90% confidence error circle of radius 30 arc s is: $\alpha = 1^{\text{h}} 15^{\text{m}} 15.7^{\text{s}}$, $\delta = 63^{\circ} 29' 45''$ (equinox 1950.0). This position agrees with the more accurate one subsequently

obtained by HEAO-1 (ref. 18). Figure 3 shows the location of both the SAS 3 error circle and the 4U (ref. 19) position superposed on a blue Palomar sky survey print.

The brightest star in the 30-arc s radius circle (star 1 in Fig. 3) has been studied by Johns *et al.*⁴ who find that its spectrum is consistent with a B-type star with an extinction of ~ 5 mag. They also report H α and possible H β emission lines. We believe this star is the likely candidate for the optical counterpart of 4U0115+63.

The models that have been proposed to explain the transient nature of X-ray sources and which invoke variable mass transfer fall into two classes. In both it is assumed that the transient sources are compact objects in binary systems. Models of one class are based on the idea that a sudden increase in activity in the optical companion in a close binary system produces episodic mass transfer^{20,21}. Such mass transfer may result from an enhanced stellar wind from an early-type star²², a variable expulsion of material from a rapidly rotating Be star²³, or a sporadic increase in the rate at which matter over-

Fig. 2 SAS 3 observation log of 4U0115+63 for the interval 6–14 January 1978. Each cross denotes a single scan across the source of ~ 30 s duration. The solid bars indicate Y-axis pointed observations during which time the source was viewed continuously except for a $\sim 1,500$ s period of Earth occultation each satellite orbit.



flows a filled critical potential lobe²⁴. In both 4U0115+63 (ref. 18) and A0535+26 (ref. 25), the absence of any extreme changes in the optical brightness of the associated stars during X-ray outbursts may place constraints on such models.

In models of the other class, eccentric binary orbits for the transients are invoked^{26,27}. Avni, Fabian, and Pringle²⁸ have studied the relationship among the eccentricities, limiting orbital periods, recurrence times, and durations of the outbursts for several transients under the assumption that the primary loses mass by a steady wind and that the source flares close to periastron passage. They showed that very large eccentricities

between spectral hardness and pulsations in X-ray stars (see refs 17, 29, 30).

We thank J. G. Jernigan, C. Canizares and H. Bradt for helpful discussions, and C. Reid and M. Johnston for assistance in preparing Fig. 3. L.C. acknowledges support from Zonta International and S.R. from the Alfred P. Sloan Foundation. This work was supported in part by the US NASA under contract NAS5-11450.

L. COMINSKY
G. W. CLARK
F. LI
W. MAYER
S. RAPPAPORT

Department of Physics and Center for Space Research,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139

2S0115+634

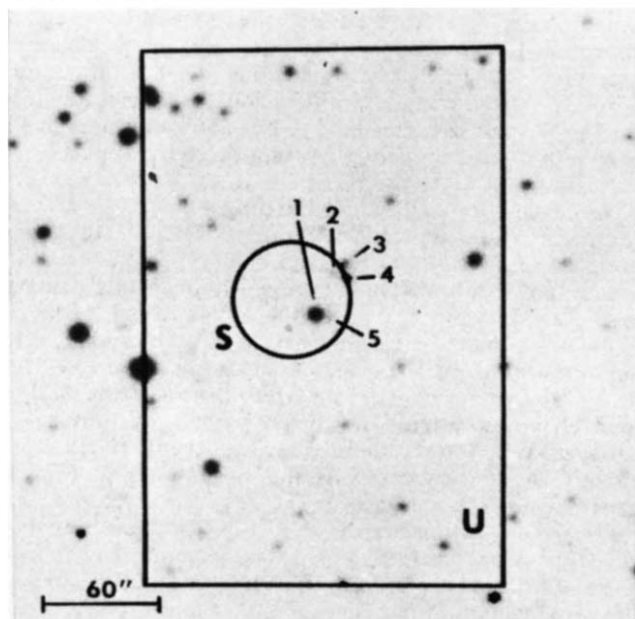


Fig. 3 The celestial location of 2S0115+634 (4U0115+63) superimposed on a print of the blue Palomar Sky Survey (©National Geographic Society). The 90% confidence SAS 3 error circle and the 4U error box are indicated by S and U, respectively. The numbers assigned to various stars are for identification purposes. The likely optical counterpart of the transient source is star 1 (see text).

($e > 0.99$) would be required to account for both the observed duration of the outbursts and their recurrence intervals, and concluded that eccentric binary orbits are unlikely to provide a complete explanation for X-ray transients.

Preliminary analysis of the X-ray Doppler curve for 4U0115+63 has already provided information on the orbital elements and mass function for this system which will be presented in a subsequent paper. Rappaport *et al.*⁵ find that 4U0115+63 is in a moderately eccentric orbit with a binary period of ~ 24 d. The time between the two reported outbursts of 4U0115+63 (~ 7 yr) is clearly not the orbital period of the system. The fact that the recurrence time is much longer than the orbital period may indicate that periastron passage must coincide with some other infrequent phenomenon to cause a rare and transient transfer of mass. This mass may then be stored in an accretion ring which gradually contracts, supplying the material for accretion by the neutron star for the duration of an outburst of X rays that may last for one or two months.

In conclusion, we have discovered a 3.61-s pulsation in the hard, recurrent transient X-ray source 4U0115+63 and have measured its position to ~ 30 arc s. A likely optical identification^{4,5} has been made on the basis of the position measurement^{3,18} and a preliminary determination of the mass function⁵. The presence of pulsations strengthens the general correlation

Received 24 February; accepted 31 March 1978.

- Forman, W., Jones, C. & Tananbaum, H. *Astrophys. J. Lett.* **206**, L29-L35 (1976).
- Clark, G. W. & Cominsky, L. *IAU Circ.* No. 3161 (1978).
- Cominsky, L., Li, F., Bradt, H., Clark, G. W. & Rappaport, S. *IAU Circ.* No. 3163 (1978).
- Johns, M., Koski, A., Canizares, C. & McClintock, J. *IAU Circ.* No. 3171 (1978).
- Rappaport, S., Clark, G., Cominsky, L. & Li, F. *IAU Circ.* No. 3171 (1978).
- Ives, J. C., Sanford, P. W. & Bell-Burnell, S. J. *Nature* **254**, 578-580 (1975).
- Rosenberg, F. D., Eyles, C. J., Skinner, G. K. & Willmore, A. P. *Nature* **256**, 628-630 (1975).
- Bradt, H. *et al. Astrophys. J. Lett.* **204**, L67-L71 (1976).
- Maraschi, L., Huckle, H. E., Ives, J. C. & Sanford, P. W. *Nature* **263**, 34-36 (1976).
- Chevalier, C. & Ilovaisky, S. *IAU Circ.* No. 2778 (1975).
- Stier, M. & Liller, W. *Astrophys. J.* **206**, 257-259 (1976).
- Rappaport, S., Joss, P. C., Bradt, H., Clark, G. W. & Jernigan, J. G. *Astrophys. J. Lett.* **208**, L119-L123 (1976).
- Holt, S. & Kaluzienski, L. J. *IAU Circ.* No. 3161 (1978).
- Lewin, W. H. G. *et al. Mon. Not. R. astr. Soc.* **177**, 93P-100P (1976).
- Buff, J. *et al. Astrophys. J.* **122**, 768-773 (1977).
- Doxsey, R. *et al. Astrophys. J. Lett.* **203**, L9-L12 (1976).
- Jones, C. *Astrophys. J.* **214**, 856-873 (1977).
- Johnston, M. *Astrophys. J. Lett.* (submitted); *IAU Circ.* No. 2163 (1978).
- Forman, W. *et al. Astrophys. J. Suppl.* (in the press).
- Bath, G. T., Evans, W. D., Papaloizou, J. & Pringle, J. C. *Mon. Not. R. astr. Soc.* **169**, 447-470 (1974).
- Osaki, Y. *Publ. astr. Soc. Japan* **26**, 429-436 (1974).
- Wickramasinghe, D. T. & Whelan, J. A. *Nature* **258**, 502-503 (1975).
- Maraschi, L., Treves, A. & van den Heuvel, E. P. J. *Nature* **259**, 292 (1976).
- Li, F. K., Sprott, G. F. & Clark, G. W. *Astrophys. J.* **203**, 187-192 (1976).
- Bartolini, C., Guarnieri, A., Piccioni, A., Giangrande, A. & Giovanelli, F. *IAU Circ.* No. 3167 (1978).
- McCluskey, G. E. & Kondo, Y. *Astrophys. Space Sci.* **10**, 464-469 (1971).
- Tsygan, A. I. *Sov. Astr.-A.J.* **18**, 798-799 (1975).
- Avni, Y., Fabian, A. C. & Pringle, J. E. *Mon. Not. R. astr. Soc.* **175**, 297-304 (1976).
- Cominsky, L., Jones, C., Forman, W. & Tananbaum, H. *Astrophys. J.* (in the press).
- Rappaport, S. *et al. Astrophys. J. Lett.* **217**, L29-L33 (1977).

Ultraviolet spectra of organic molecules and the interstellar medium

THE polysaccharides cellulose and starch have been shown to give an excellent match to certain spectra of the interstellar medium¹. The matching was based on comparisons of the laboratory spectra of polysaccharides in the 2-30- μ m region with those of various astronomical objects, in particular dark clouds near the Trapezium^{2,3}. Cellulose has broad absorption bands at 3, 10 and 20 μ m devoid of the 10.3- μ m high transmittance spike reported for crystalline silicates⁴, the commonly accepted dominant interstellar material. It has been claimed that cellulose obviates the need for the presence of both H₂O ice and silicates in the interstellar medium¹. Several organic molecules have already been found associated with dust grains in the interstellar medium. The dust grains offer protection from dissociation and could aid in the cellulose formation process⁵. The ultraviolet (UV) absorption in the interstellar medium can provide an additional identification technique for interstellar organic molecules, and this poses the question as to what are the UV spectral characteristics of cellulose. We have tried to answer this question by measuring the UV spectrum of cellulose between 0.19 and 0.40 μ m, and com-

paring to the observed UV spectrum of the interstellar medium⁶. The sample we chose was regenerated cellulose produced from wood pulp, known generally as cellophane.

The UV absorption spectrum for a 1 mil (0.025 μm) film was made on a Cary 14 spectrophotometer and is shown in Fig. 1. The transmission (expressed as optical density) is shown as a function of wavelength between 0.19 and 0.40 μm . A scale change takes place at the left of the figure where the density range is then 1.0 to 2.0. It can be seen that the transmission is high from 0.40 μm to about 0.36 μm (~90%), dropping slowly to shorter wavelengths, with a sharp drop in transmission beginning at 0.23 μm , and a transmission of 1% at 0.19 μm . There is no evidence of the extinction feature at 0.22 μm that is observed for the interstellar medium⁶. However, the opacity remains high toward shorter wavelengths, in agreement with interstellar medium observations⁶.

In the infrared (IR) region, published spectra of cotton (cellulose) and other polysaccharides such as amylose, arabinogalactan and mannan dispersed in KBr (ref. 7) are limited by having uncalibrated absorption coefficients. That is, scattering is not separated from absorption. This separation could be accomplished by use of additional measurements and a suitable theoretical approach⁸. However, using cellophane precludes the occurrence of significant scattering, and eliminates this limitation. An IR spectrum of the same cellophane sample as used for our UV measurements was taken on a Perkin Elmer 457 spectrophotometer in the region between 2.5 μm and 40 μm (Fig. 2) and confirms it to be cellulose. The percent transmittance is shown as a function of wavelength (wavenumbers). Interference fringes are evident at the shorter wavelengths, apparently the result of using different optical systems.

A calculation of the absorption coefficient at 3 μm and 10 μm may be made for the cellophane sample by scaling our observed absorption at 3.4 μm (Fig. 2) to that at 3 μm and 10 μm using previously published data on a lower absorbing sample⁷. The results are that the absorption coefficient, α , is 870 cm^{-1} at both 3 and 10 μm .

Although the IR spectrum of cellulose is a good match to several observations of the interstellar medium¹, we feel that there are still arguments against its being a dominant constituent. First, recent absorption spectrum measurements of amorphous silicates⁹ have shown a much better match to the 10- μm interstellar feature than previous measurements of crystalline silicates. The undesirable sharp transmission spike is not present for the amorphous form. There also remains the problem of explaining the extremely wide variation (0- ∞) in the ratio of the strength of the 3-10- μm absorption bands observed in the interstellar medium¹⁰. Such a range of variation in band strength is difficult to account for by temperature and absorption variations, and suggests at least a two-component medium. Further, late M-type stars constantly eject large quantities of silicates from their atmospheres¹¹. These must then contribute to interstellar absorption unless some as yet unknown mechanism is capable of destroying silicates.

Fig. 1 UV transmission curve for 1 mil (0.025 mm) thickness of cellophane.

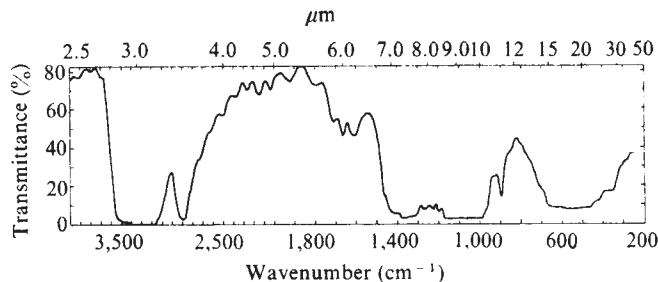
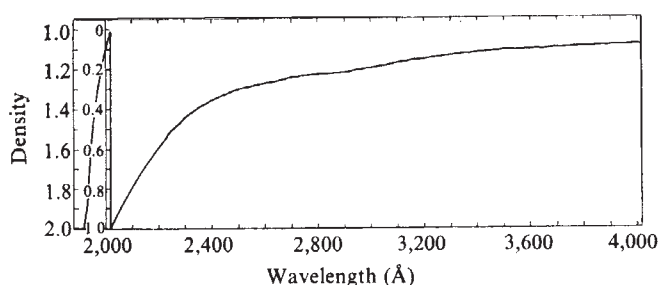


Fig. 2 IR transmittance curve for 1 mil (0.025 mm) thickness of cellophane.

Finally, the UV measurements in Fig. 1 have shown that we need something other than cellulose in the interstellar medium to account for the 0.22- μm extinction feature. This absorption has previously been postulated as produced by three processes; by plasma oscillations in solids (graphite)¹²; by solids having sharp UV absorption edges (silicates (Mg, Fe) SiO_3)¹³; and by solids having UV absorption features in their bulk optical properties (irradiated quartz¹⁴; plagioclase feldspars¹⁵).

We suggest another possibility for the 2,200-Å feature. Consistent with the reported radio detection of cyano-octatetrayne (C_8HN) in Heik's Cloud 2¹⁶ is the presence of a cyano or nitrile (C-N) group. Although this molecule has never been synthesised in the laboratory, it is known that similar bonded molecules have a UV absorption in the appropriate region. For instance methacrylonitrile ($\text{C}_5\text{H}_7\text{N}$) has an absorption at 0.215 μm (ref. 17), near the 0.22 μm interstellar absorption. The nitrile group may thus be the cause of a UV absorption feature at the required wavelength. Another possibility is the carbonyl bond (C=O) which has an absorption near the required spectral region. For instance, formaldehyde, H_2CO , which has been confirmed as existing in the interstellar medium¹⁸, has a strong absorption band at 1,850 Å (ref. 19). The actual location of the carbonyl absorption depends on the structure of the organic molecule with which it is associated. It has been noted that there is an underabundance of C, N, and O in the interstellar gas²⁰ and the presence of condensed organics may account for these missing elements.

We feel that although cellulose (or other organics) might exist in the interstellar medium, we are not yet prepared to reject the presence of H_2O ice and silicates.

We thank N. C. Wickramasinghe, J. M. MacLeod, R. Delasi, L. L. Smith and J. Krassner for helpful discussions.

W. G. EGAN
T. HILGEMAN

Research Department,
Grumman Aerospace Corporation,
Bethpage, New York 11714

Received 7 March; accepted 22 March 1978.

1. Hoyle, F. & Wickramasinghe, N. C. *Nature* **268**, 610 (1977).
2. Forrest, W. J. & Soifer, B. T. *Astrophys. J.* **208**, L129 (1976).
3. Forrest, W. J., Houck, J. R. & Reed, R. A. *Astrophys. J.* **208**, L133 (1976).
4. Day, K. L. *Astrophys. J.* **199**, 660 (1975).
5. Goldanskii, V. I. *Nature* **268**, 612 (1977).
6. Stecher, T. P. *Astrophys. J.* **157**, L125 (1969).
7. Hoyle, F., Olavesen, A. H. & Wickramasinghe, N. C. *Nature* **271**, 229 (1978).
8. Egan, W. G. & Hilgeman, T. *Icarus* **25**, 344 (1975).
9. Day, K. L. *Astrophys. J.* **210**, 614 (1976).
10. Merrill, K. M., Russell, R. W. & Soifer, B. T. *Astrophys. J.* **207**, 763 (1976).
11. Gehry, R. & Woolf, N. *Astrophys. J.* **165**, 285 (1971).
12. Gilra, D. P. *NASA SP-310*, 295 (1972).
13. Huffman, D. R. & Stapp, J. L. *Nature* **229**, 45 (1971).
14. Wickramasinghe, N. C. *Nature* **234**, 7 (1971).
15. Egan, W. G. & Hilgeman, T. *Astr. J.* **80**, 587 (1975).
16. Broten, N. W., Avery, L. W., MacLeod, J. M., Oka, T. & Kroto, H. W. *Pap. 151st mtg Am. Astr. Soc. Austin, Texas* (1978).
17. Dyer, J. R. *Applications of Absorption Spectroscopy of Organic Compounds* (Prentice-Hall, Englewood Cliffs, N.J., 1965).
18. Dickel, H. R., Seacord, A. W. II, & Gottesman, S. T. *Astrophys. J.* **218**, 133 (1977).
19. Jaffe, H. H. & Orchin, M. *Theory and Applications of Ultraviolet Spectroscopy* (Wiley, New York, 1962).
20. Greenberg, J. M. *Astrophys. J.* **186**, L81 (1974).

Cooperativity in the dislocation theory of melting

THE interesting behaviour of the shear moduli of alkali halides in the vicinity of the melting transition was recently noted by Tallon, Robinson and Smedley¹. These parameters do not fall to zero at the crystal melting temperature, T_m , as proposed by Born², but a zero value is attained on extrapolating to the dilation corresponding to the liquid at T_m . It is suggested that 'melting occurs when a solid can transform isothermally to a state of zero shear modulus', and that a two-phase melting model follows. Although they do not deal with the microscopic processes occurring along the melting isotherm their discussion does have a bearing on the important role of cooperative effects. We comment here both on the specific points raised by their analysis and the question of cooperativity in the dislocation theory of melting.

The extrapolation by Tallon *et al.* would not be possible for crystals showing a negative volume change of melting. Such materials, nevertheless, exhibit continuous expansion right up to T_m . Also a plot of shear modulus against volume causes temperature to become a 'hidden variable' for the region below T_m . The success of the Tallon *et al.* extrapolation suggests, however, that, for the alkali halides at least, the volume change is the dominating influence on the shear modulus.

Mizushima³ (and recently Edwards⁴) showed that because dislocation energy, E_d , depends on dislocation concentration, C_d , the free energy of a crystal will vary with C_d (used as an order parameter) in the manner described by Landau and Lifschitz⁵ for a first order transition; for $T < T_m$ equilibrium prevails at $C_d = 0$, but C_d suddenly goes to its saturation value at $T = T_m$. Further insight into the physical basis of this behaviour is obtained by noting⁶ that at equilibrium $C_d = \exp [-(E_d - TS_d)/kT]$, S_d being dislocation entropy, but that $E_d = -(Gb^2/8\pi)\ln C_d\delta^2$, where $\delta < 1$ and C_d is given as an atomic fraction⁷, (G and b are the shear modulus and Burgers vector, respectively). Thus, C_d depends on T in a 'super-Arrhenius' manner, having a non-zero equilibrium value only at a temperature $T^* = -b^3G(\ln \delta^2)/40\pi k$, the limit of stability in the infinite crystal, which Mizushima identifies as T_m . Kuhlmann-Wilsdorf has extended this approach and shown that it leads to impressive agreement between experimental melting points and values calculated from elastic constants and crystallographic data⁸. Taking a lead from Tallon *et al.*¹, we point out that because a dislocated lattice dilates⁹, G will fall as C_d increases. This additional factor will make C_d depend even more critically on T in the vicinity of T^* . Moreover, dilation propagates through the crystal at the sound velocity so this cooperativity is potentially stronger than the essentially positional factor arising in Mizushima's model. Finally, even S_d will vary with C_d through dilation-induced changes in the eigen frequencies.

Dislocations generated inside a crystal by thermal fluctuations, either at or below T_m , must, from topological requirements, be dipoles ($+b$ and $-b$) as predicted by Kuhlmann-Wilsdorf⁸, and subsequently observed^{10,11}. These are favoured by the C_d dependence of E_d because the two dislocations cancel each other's strain field and only the core energy is left (that is, E_d simply becomes the core energy). This only applies as long as they are a bound pair, so the inevitable fate of a dipole below T_m is mutual annihilation. Furthermore, the influence of such transient dipoles on a crystal's viscosity is not just small but identically zero because the effect of one dislocation on the lattice will always be exactly counteracted by the second member of the dipole pair. The saturation (and instability) situation is reached when the inter-dipole distance equals the spacing of the dislocations in the dipoles (about a core diameter), which is analogous to the Debye screening length⁴.

In practice, crystals melt at their surfaces, and the influence of a bounding interface (solid-gas or solid-liquid) has been discussed in both continuum¹² and atomistic^{13,14} models. In particular, Tallon¹³ shows how a surface can interfere with

mutual annihilation in dipole pairs by absorbing a dislocation. Energetic considerations also show that the surface functions as a dislocation source and becomes a natural nucleation site for the cooperative process discussed above. The surface has, in effect, a melting point, T_{sm} , which lies lower than Mizushima's T^* , and corresponds to the situation in which the dislocation saturation condition is attained in a layer of minimal thickness (presumably equal to the dislocation core diameter). The picture of melting that emerges from these considerations is as follows: between T_{sm} and T^* dynamic equilibrium between dislocation generation and annihilation leaves the interior dislocation free but permits a gradual rise to the saturation dislocation density at the surface. Immediately above T^* the crystal-liquid interface advances into the interior at a speed that is predominantly determined by the dipole generation rate.

R. M. J. COTTERILL

*Department of Structural Properties of Materials,
The Technical University of Denmark,
DK-2800 Lyngby, Denmark*

Received 18 November 1977; accepted 5 April 1978.

1. Tallon, J. L., Robinson, W. H. & Smedley, S. I. *Nature* **266**, 337 (1977).
2. Born, M. *J. chem. Phys.* **7**, 591 (1939).
3. Mizushima, S. *J. phys. Soc. Japan* **15**, 70 (1960).
4. Edwards, S. F. *Polymer* **17**, 933 (1976).
5. Landau, L. D. & Lifschitz, E. M. *Statistical Physics*, 426 (Pergamon, Oxford, 1970).
6. Friedel, J. *Dislocations*, 75 (Pergamon, Oxford, 1964).
7. Nabarro, F. R. N. *Theory of Crystal Dislocations*, 688 (Oxford University Press, 1967).
8. Kuhlmann-Wilsdorf, D. *Phys. Rev.* **145**, 465 (1966).
9. Nabarro, F. R. N. *Theory of Crystal Dislocations*, 166 (Oxford University Press, 1967).
10. Cotterill, R. M. J. & Pedersen, L. B. *Solid St. Commun.* **10**, 439 (1972).
11. Cotterill, R. M. J., Damgaard Kristensen, W. & Jensen, E. *J. Phil. Mag.* **30**, 245 (1974).
12. Klæstrup Kristensen, J. & Cotterill, R. M. *J. Phil. Mag.* **36**, 437 (1977).
13. Tallon, J. L. thesis, Victoria Univ. Wellington, New Zealand (1976).
14. Cotterill, R. M. *J. Phil. Mag.* **32**, 1283 (1975).

Uncatalysed oscillatory chemical reactions

THE most widely studied oscillatory chemical reaction is the catalytic oxidation of a variety of organic compounds by acidic bromate—the Belousov-Zhabotinsky reaction. Babu *et al.*¹ have reported that the gallic acid, bromate, sulphuric acid and cobalt (II) system showed oscillatory behaviour. They claim that the two key steps are the oxidation of cobalt (II) by bromate to cobalt (III), and the reduction of the latter by gallic acid (GA), that is, they regarded the chemical process as a Belousov-Zhabotinsky-type oscillatory reaction. Redox potential data on the $\text{Co}^{3+}/\text{Co}^{2+}$ (1.81 V), $\text{BrO}_3^-/\text{Br}^-$ (1.44 V) and $\text{BrO}_3^-/\text{Br}_2$ (1.51 V) systems, imply that the reduction of bromate by cobalt (II) is highly unfavourable thermodynamically which made us sceptical about the suggested steps. We report here our preliminary investigations mostly on the $\text{GA}-\text{BrO}_3^--\text{H}_2\text{SO}_4$ system which attempted to unravel this problem. One of our first experiments showed that chemical oscillation occurred even in the absence of a catalyst. This unexpected finding indicated novel perspectives regarding chemical oscillators.

Adding bromate to a stirred mixture of GA and H_2SO_4 immediately turns the solution dark brown, and after a certain time suddenly to yellow. This is followed by repeated colour changes from brown to yellow with a decreasing frequency. The oscillatory behaviour of the system was traced with a Pt electrode or with a bromide selective sensor; $\text{Hg}/\text{Hg}_2\text{SO}_4/\text{K}_2\text{SO}_4$ was used as a reference electrode. Periodicity was found also in the rate of heat evolution similar to that observed with the Belousov-Zhabotinsky systems²⁻⁴. Chemical oscillations were looked at in the following concentration ranges: 0.025–0.05 M GA, 0.04–0.075 M KBrO_3 , and above 1.5 M H_2SO_4 and 1.5 M HNO_3 , respectively. (All the chemicals used were of analytical grade with heavy metal content below 5 p.p.m. checked by atomic absorption spectrophotometry.) Typical curves of redox potential and bromide concentration oscillations are shown in Fig. 1. The temperature change during the reaction is seen in Fig. 2. A few data on the length of time of the first

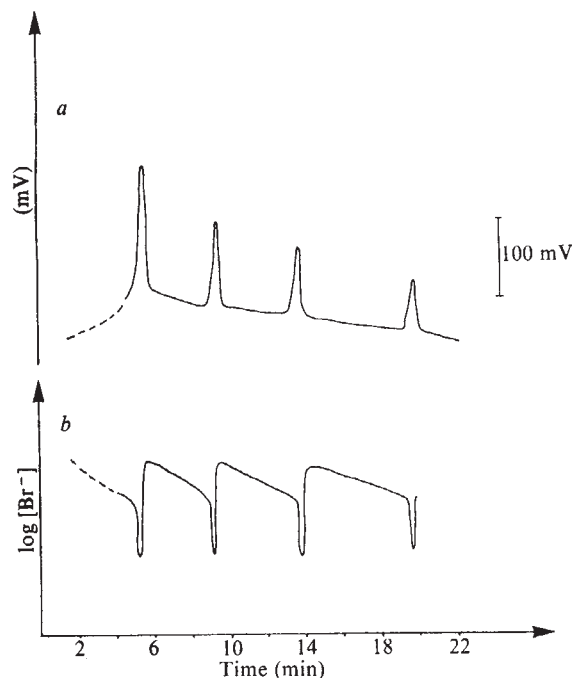


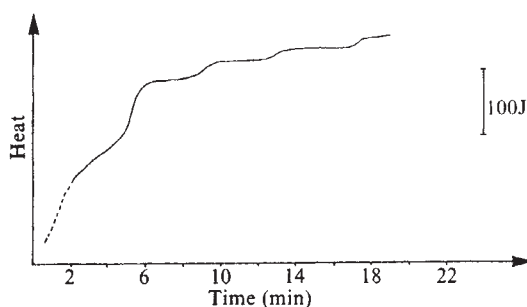
Fig. 1 Redox potential (a) and log Br⁻ concentration (b), against time curves at 25 °C. The chemical system containing GA (0.05 M), KBrO₃ (0.075 M) and H₂SO₄ (2.0 M).

two cycles at different reagent concentrations and at 20.5 °C are given in Table 1.

By adding bromide ion (about 10⁻³ M) to the reaction mixture the frequency of that particular period decreased, but no change was observed in the consecutive cycles. The chemical oscillation was inhibited by chloride ion in concentrations higher than 10⁻⁴ M. The activation energy⁵ of the oscillatory process in a system of 0.025 M GA, 0.075 M KBrO₃ and 2.0 M H₂SO₄ is 67 kJ mol⁻¹ measured in the temperature range of 288.3–313 K. Metal ions (in 10⁻³–10⁻⁴ M concentrations) have the following effects: Co²⁺ is ineffective, Mn²⁺ which is a Belousov-Zhabotinsky catalyst increases the potential jumps dramatically, and Fe²⁺ blocks the oscillation. The latter is oxidised by bromate and Fe³⁺ reacts with GA producing a blue-black solution. (The GA-BrO₃⁻-H₂SO₄-Ce³⁺ system has been investigated by Babu *et al.*⁶) Bromate cannot be replaced by other oxidising oxianions, such as ClO₃⁻, IO₃⁻, MnO₄⁻, Cr₂O₇²⁻.

One explanation for the occurrence of chemical oscillation in the GA-BrO₃⁻-H₂SO₄ system, consistent with our experimental findings is that in strongly acidic solution bromate oxidises GA mainly to ellagic acid, that is, by coupling two benzene rings. However, in appropriate conditions the overall reaction proceeds in two major irreversible steps: the irreversibility of the first step was proved by cyclic voltametry. First,

Fig. 2 A typical temperature against time curve. The chemical system containing GA (0.025 M), KBrO₃ (0.10 M) and H₂SO₄ (2.0 M).



from GA a dark compound, most likely a quinone-type molecule, is formed, and bromate is reduced to bromide. The latter is an inhibitor for the further oxidation of the quinone-type compound by bromate. When the bromide concentration drops below a critical value due to the bromate-bromide reaction, the second oxidation step switches on, resulting in a yellow solution. Bromide is formed again which does not, however, inhibit the first oxidation step. The brown colour develops again. The cycle repeats periodically with decreasing frequency and amplitude until GA is consumed. At most, 15 oscillations were observed.

The oscillatory oxidation of GA by acidic bromate has many similarities to the Belousov-Zhabotinsky reaction⁷: (1) the kinetic states of the system are controlled by the bromide ion concentration; (2) chloride is an effective inhibitor; and (3) the activation energy falls within the range characteristic for Belousov-Zhabotinsky reactions—between 65 and 75 kJ mol⁻¹ (refs 5, 8, 9).

Table 1 Time and chemical concentration data

Chemical composition of the oscillatory system			Time (min)	
GA (M)	KBrO ₃ (M)	H ₂ SO ₄ (M)	1st Cycle	2nd Cycle
0.025	0.075	2.0	3.44	1.64
0.025	0.075	1.5	4.36	3.24
0.025	0.075	1.0	No oscillation	
0.025	0.050	2.0	5.84	5.12
0.025	0.0375	2.0	15.2	Not measured
0.0125	0.075	2.0	No oscillation for 30 min	

All experiments were performed using deaerated solutions and in N₂-atmosphere. Later it was found that this precaution was unnecessary.

Experimental results obtained from the GA, bromate and sulphuric acid system indicate that a series of redox reactions are involved in the oscillatory process; balancing them leads to the development of temporal chemical periodicity. It seems probable that if an organic compound is oxidised in two basic steps the product of the first is relatively stable, while the next step will be bromide inhibited; the overall process will exhibit periodicity—the organic compound will be oxidised in discrete portions to the final product. The quantity of each portion depends on the chemical composition and the temperature of the system.

This conception has prompted us to search for further oscillatory systems. Experiments performed to date indicate that chemical oscillation is not a rare phenomenon when poly-substituted aromatic compounds are oxidised by acidic bromate. Potential oscillations were recorded during the oxidation of pyrogallol, hydroxyquinone, resorcinol and tannic acid. However, the oxidation of hydroquinone, pyrocatechol and phloroglucinol does not proceed in an oscillatory manner. Another candidate for such chemical oscillation is the reaction mixture of an aryldiazonium salt, bromate and sulphuric acid which shows sustained temporal chemical periodicity¹⁰.

E. KÖRÖS
M. ORBÁN

*Institute of Inorganic and Analytical Chemistry,
L. Eötvös University,
H-1443 Budapest, PO Box 123,
Hungary*

Received 17 January; accepted 20 March 1978.

- Babu, J. S. & Srinivasulu, K. *Proc. Ind. natn. Sci. Acad.* **42**, 361–363 (1976).
- Körös, E., Orbán, M. & Nagy, Zs. *Nature phys. Sci.* **242**, 30–31 (1973); *J. phys. Chem.* **77**, 3122–3123 (1973).
- Janjic, D., Stroot, Ph. & Burger, U. *Helv. chim. Acta* **56**, 266–271 (1974).
- Körös, E. *et al. Faraday Symp. Chem. Soc. No. 9*, 28–37 (1974).
- Körös, E. *Nature* **251**, 703–704 (1974).
- Babu, J. S., Bokadi, M. M. & Srinivasulu, K. *Bull. chem. Soc. Jap.* **49**, 2875–2876 (1976).
- Field, R. J., Körös, E. & Noyes, R. M. *J. Am. chem. Soc.* **94**, 8649–8665 (1972).
- Blandamer, M. J. & Morris, S. H. *Faraday Trans. 1* **71**, 2319–2330 (1975).
- Farage, V. J., Stroot, Ph. & Janjic, D. *Helv. chim. Acta* **60**, 231–236 (1977).
- Kuhnert, L. & Linde, H. *Z. Chem.* **17**, 19–20 (1977).

Silicate in anoxic pore waters and oxidation effects during sampling

SILICA concentrations in pore fluids of sediments, and the exchange between pore water and the overlying water are important considerations for the silica budgets of the marine environment¹⁻³. Recent studies have shown, however, that inadequate sampling and handling procedures of marine sediments can produce large compositional changes in the pore fluids of these sediments⁴⁻⁶. Changes in sediment temperatures, similar to those occurring during sample acquisition, can alter the pore water silica concentration by as much as 50% (ref. 7). Oxidation may also produce artefacts in pore water data, that is oxidation of iron-rich anoxic pore waters has been shown to decrease the concentration of iron and phosphate in estuarine pore waters^{8,9}. We present here laboratory data which indicate that the exposure of anoxic pore waters to the ordinary atmosphere can reduce the silica concentration by approximately 15%, which may reduce the reported diffusional fluxes of silica from anoxic marine sediment by 15%.

Three cores (cores 1-3) were taken during August 1976, and one core (core 4) in October 1976, from a shallow sub-tidal area in the Great Bay Estuary, New Hampshire. The samples were collected with a corer consisting of 15 2-cm segments of 6.6-cm diameter polycarbonate core liner cleaned and taped together with electrical tape (D. Schink, personal communication). Cores were obtained by pushing the corer into the sediment, placing a stopper on the open end, and carefully removing the corer.

The cores were treated very carefully so that the sediment would not be exposed to the atmosphere at any time. After sampling, the ends were purged with nitrogen gas and stoppered, and the corer was returned to the laboratory in a polyvinyl chloride (PVC) core carrier also purged with nitrogen¹⁰. All sample processing was carried out in a nitrogen-purged glove bag. The corer was disassembled in an upright position by removing the tape and cutting the sediment one 2-cm segment at a time with a teflon coated spatula, starting at the top. Half of each segment was centrifuged under nitrogen in the glove bag. The other half was stored in a plastic beaker under nitrogen, transferred out of the glove bag and centrifuged in the laboratory atmosphere. Approximately 2-3 ml of pore water were obtained from each half of each 2-cm segment—enough for analysis of reactive silicate, phosphate, and iron.

Sediment samples for cores 1-3 were processed at *in situ* temperatures ($\pm 3^\circ\text{C}$) while samples from core 4 were processed at about 9°C higher. The temperature effects were the same for all cores because the air and nitrogen samples were processed at the same temperature. However, absolute silica values for core 4 are probably 20% too high⁷.

After centrifugation each pore water sample was filtered using a 2.5-cm diameter, 0.4- μm Nuclepore filter in a Millipore Swinnex-25 filter holder under nitrogen or in air as appropriate. The samples were then placed in acid-washed, 30-ml conventional polyethylene (CPE) bottles, acidified with 100- μl of 3.1 M Baker Ultrex HCl and frozen until analysis during the following 2-week period. This storage by freezing has no effect on silica if the sample salinity is greater than 27‰ or if samples have been allowed to stand for several hours¹¹. As our samples had salinities of 20-32‰ they were allowed to stand for several hours before analysis. Samples processed in the nitrogen atmosphere were first exposed to air after centrifugation and filtration while the other set were exposed to air for 10 to 12 min during centrifugation and filtering before acidification. All samples were centrifuged for 10 min and any warming due to this process was similar for both sets of samples. It was found that once acidification was completed, the pore water

Table 1 Decrease in interstitial silica and phosphate concentrations for samples processed in laboratory atmosphere compared with a nitrogen atmosphere for cores from the Great Bay Estuary

Core	No. of samples	% decrease in silica		% decrease in phosphate	
		Average	Range	Average	Range
1	9	13.0	(1.4-20.4)	No data	
2	8	10.0	(3.3-25.0)	36.4	(13.6-70.8)
3	11	15.6	(8.5-41.1)	No data	
4	8	10.0	(3.8-19.7)	43.5	(13.8-65.0)

Data from the 0-2 cm segment are not included since sediments in this segment were not anoxic.

samples could be exposed to air with no loss of iron, phosphate or silicate.

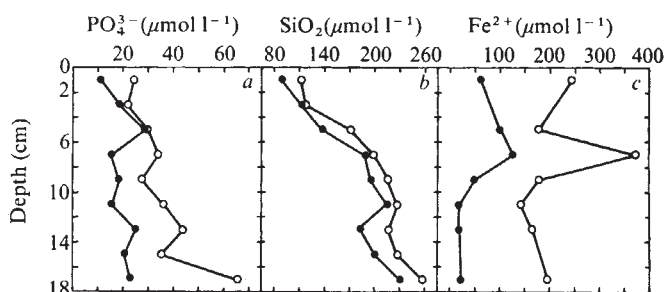
Nutrient analyses were run on a Technicon Auto-Analyzer II system using Technicon's methods for silica¹² and phosphate¹³. Portions (100- μl) of each sample were placed in 5-ml plastic sample cups and diluted 11:1 for silicate and 6:1 for phosphate. The average difference between analyses of duplicate dilutions of pore waters for core 4 was 1.6% for silica and 1.5% for phosphate. Total iron was analysed by the ferrozine method¹⁴ modified for small volume natural water samples by Murray and Gill¹⁵ with a standard deviation of $\pm 0.1 \mu\text{M l}^{-1}$.

We found that there was an average decrease in reactive silicate of 10-15.6% when samples were exposed to laboratory air before centrifugation and filtration compared to samples processed under nitrogen. The variability of this decrease (1-25%) was probably due to the fact that the amount of exposure to air varied somewhat for each sample. Phosphate data for the same samples (Table 1, Fig. 1) show an even greater proportional decrease.

Our iron data from core 4 indicates that an average of 75% of the Fe^{2+} in the pore waters of the Great Bay anoxic sediments was removed by as short as a 12-min exposure to the laboratory atmosphere. These data agree with those of Troup *et al.*⁸, indicating that much care should be taken if valid Fe^{2+} data are to be obtained from similar samples. Our phosphate data support the findings of Bray *et al.*⁸ suggesting that dissolved phosphate is removed from anoxic waters either by the precipitation of a ferric phosphate mineral phase or by chemisorption on to the ferric hydroxide.

The mechanism involved in the removal of dissolved SiO_2 from the pore fluids is undoubtedly related to the oxidation of Fe^{2+} to Fe^{3+} which proceeds very rapidly at neutral pH. The Fe^{3+} forms amorphous hydroxides which are capable of

Fig. 1 Effects of pore water removal under nitrogen atmosphere (○) and normal laboratory atmosphere (air) (●) on the concentration of phosphate (PO_4^{3-}) (a); silicate (SiO_2) (b); and ferrous iron (Fe^{2+}) (c) for core 4 from the Great Bay Estuary, New Hampshire. Phosphate and silicate analyses were run in duplicate on the pore water extracted from 2-cm core segments of the core.



coprecipitating dissolved silicate from very dilute solutions¹⁷. Lal *et al.*¹⁸ have shown that from 12 to 15% of dissolved silicate can be removed from seawater by scavenging during precipitation of finely dispersed ferric hydroxide. Kato¹⁹ has shown that hydrated oxides of iron can remove large quantities of dissolved silica from lake water. Poldoski and Glass's²⁰ work on the pore waters of Lake Superior sediments indicates that the phenomenon of silicate loss from anoxic pore fluids on interaction with oxygen occurs in fresh water as well. Their data also show a dramatic loss of iron and phosphorus. Therefore, the removal of these dissolved chemical constituents from anoxic pore waters seems to be a common phenomenon and not confined to salt water.

We have calculated the typical amount of dissolved iron, phosphate and silicate removed by this oxidation effect. For every 8- μ M of Fe^{2+} that is oxidised to Fe^{3+} and removed from solution approximately 1- μ M of silicate and 0.7 μ M of phosphate are also removed. If strengite, a ferric phosphate mineral, was forming upon oxidation, the Fe^{2+} to PO_4^{3-} ratio would be 1:1 instead of the 11:1 that we have observed. Likewise, if nontronite, the most likely iron-silicate mineral to form¹⁷ was precipitating, the Fe^{2+} to SiO_2 ratio would be approximately 1:2 instead of our observed value of 8:1. The observed Fe:SiO₂ ratio of 8:1 is similar stoichiometrically to one of several amorphous ferroaluminium silicates described by Nriagu²¹ in surficial Great Lake sediments. These data suggest that phosphate and silicate are probably removed by adsorption on to an amorphous ferric hydroxide phase or that silicate and iron are removed as a ferroaluminium silicate and phosphate is adsorbed to it.

As newly formed amorphous iron hydroxides seem to scavenge phosphate and silicate from solution; this process may scavenge other pore water species as well. Iron hydroxides are very good scavengers of trace metals²², and have also been used for the removal and concentration of various dissolved organic materials from seawater²³. The exposure of iron-rich anoxic waters to even small amounts of oxygen probably affects the concentrations of trace metals and dissolved organic carbon in these waters.

Fanning and Pilson⁷ emphasised that to improve the estimate of the geochemical flux of dissolved silicate from oceanic sediments, more data free of sampling artefacts must be obtained. Our data indicate that in addition to temperature-induced sampling artefacts, artefacts due to oxidation of reduced pore water species may produce decreases in silicate pore water concentrations. Therefore, we conclude that published pore water silicate data and calculated silicate diffusional fluxes from highly anoxic sediments in areas such as estuarine and hemipelagic environments may be 10–15% too low unless proper care was taken during initial sample processing.

We thank H. E. Gaudette, P. M. Glibert, M. E. Q. Pilson, C. S. Martens, and K. A. Fanning for helpful criticism. This work (contribution No. UNH-SG-JR-111 and U.N.H. CREAM No. 11) was supported by grants from the NSF-URP (SM176-03495), Sea Grant (4-20237 R/EM-2) and the UNH-Hubbard Marine Program Fund.

T. C. LODER
W. B. LYONS
S. MURRAY*
H. D. MCGUINNESS†

Department of Earth Sciences,
University of New Hampshire,
Durham, New Hampshire 03824

Received 20 February; accepted 29 March 1978.

*Present address: Bigelow Laboratory, West Boothbay Harbor, Maine 04575.

†Present address: Dept of Chemistry, Brown University, Providence, Rhode Island 02912.

1. Burton, J. D. & Liss, P. S. *Geochim. cosmochim. Acta* **37**, 1761–1773 (1973).

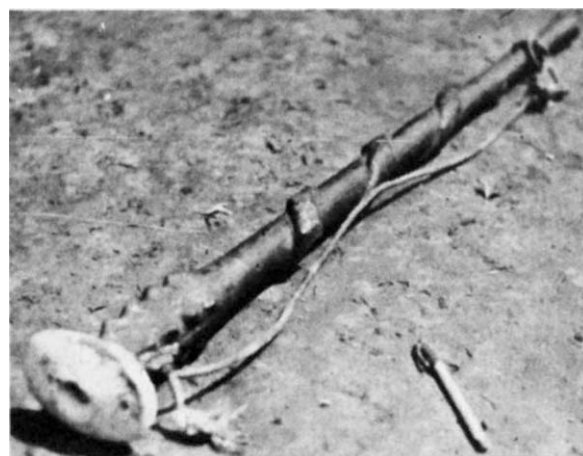
2. Heath, G. R. *Studies in Paleo-oceanography* 77–93 (Soc. Econ. Paleo. Min., 1974).
3. Schink, D. R., Guinasso, N. L. Jr & Fanning, K. A. *J. geophys. Res.* **80**, 3013–3031 (1975).
4. Bischoff, J. J., Greer, R. E. & Luistro, A. O. *Science* **167**, 1245–1245 (1970).
5. Mangelsdorf, P. C., Wilson, T. R. S. & Daniell, E. *Science* **165**, 171–174 (1969).
6. Hurd, D. C. & Spielvogel, L. Q. *J. geophys. Res.* **80**, 4975–4977 (1975).
7. Fanning, K. A. & Pilson, M. E. Q. *Science* **173**, 1228–1230 (1971).
8. Bray, J. T., Bricker, O. P. & Troup, B. N. *Science* **180**, 1362–1364 (1973).
9. Troup, B. N., Bricker, O. P. & Bray, J. T. *Nature* **249**, 237–239 (1974).
10. Lyons, W. B. & Fitzgerald, W. F. *Proc. 3rd int. Symp. Envir. Biogeochem.* (in the press).
11. Burton, J. D., Leatherland, T. M. & Liss, P. S. *Limnol. Oceanogr.* **15**, 473–476 (1970).
12. Technicon Industrial Systems, *Indus. Meth. no. 186-72W* (Tarrytown, New York, 1973).
13. Technicon Industrial Systems, *Indus. Meth. no. 155-71W* (Tarrytown, New York, 1973).
14. Stookey, L. L. *Analyt. Chem.* **42**, 779–781 (1970).
15. Murray, J. W. & Gill, G. *Geochim. cosmochim. Acta* **42**, 9–19 (1978).
16. Stumm, W. & Lee, C. F. *Indus. Engng. Chem.* **53**, 143 (1961).
17. Harder, H. *Chem. Geol.* **18**, 169–180 (1976).
18. Lal, D., Arnold, J. R. & Somayajulu, B. L. K. *Geochim. cosmochim. Acta* **28**, 1111–1117 (1964).
19. Kato, K. *Geochem. J.* **3**, 87–97 (1969).
20. Poldoski, J. E. & Glass, G. E. *Natn. Bur. Stand. Spec. Publ.* **422**, 1073–1088 (1976).
21. Nriagu, J. O. *Limnol. Oceanogr.* **23**, 53–67 (1978).
22. Jenne, E. A. *Adv. Chem. Ser.* **73**, *Am. chem. Soc.* 337–387 (1968).
23. Williams, P. M. *Nature* **189**, 219–220 (1961).

Fungal club-heads in Papua New Guinea

ALTHOUGH the ethnomycology of the native peoples of New Guinea is poorly documented it is known that throughout the island various fungi are gathered for food¹ and that certain fungi, principally mushrooms of the genus *Boletus*, play an important part in the 'mushroom madness' characteristic of the Kuma people of the New Guinea Highlands². We report here that sclerotia of *Lentinus tuber-regium* are made into club-heads by the Gogodala people who live mostly along the swampy middle reaches of the Aramia River in the Western Province of Papua New Guinea.

The club-heads are obtained from within the large mounds which birds of the family Megapodiidae construct in the local forests to incubate their eggs. The fungal material (called *utiyani*) is dried and then carved into either a smoothly rounded (Fig. 1) or a toothed disk always with a central hole to facilitate attachment to the wooden club. The carved club-heads vary from 15 cm to less than 7 cm in diameter. Specimens, now deposited with the herbarium, University of Papua New Guinea, were obtained at Isago Village (8° S, 142° 40' E), one of the more traditional Gogodala villages and the only village still occupying an old style multi-family longhouse. Uncarved specimens were irregular in shape, dark brown to black in colour; one specimen weighed 160 g and had dimensions of 79 × 68 × 54 mm. This specimen was sawn into pieces, vacuum infiltrated with

Fig. 1 Fungal club-head attached to a wooden club.



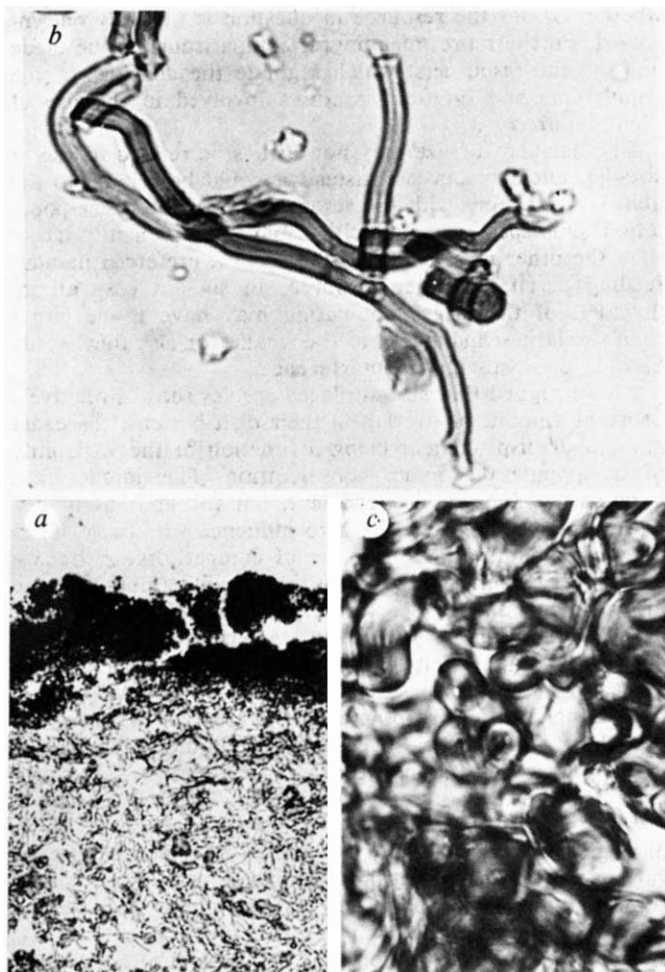


Fig. 2 a, Section of uncarved club-head showing external rind, filamentous hyphae and central core. b, Filamentous hyphae. c, Rounded, thick-walled prosenchymatous cells from central core of club-head.

formalin acetic acid, embedded in wax, sectioned, stained with ammoniacal Congo red or light green and examined microscopically. Sections consisted of an external dark coloured rind about $30\text{ }\mu\text{m}$ thick surrounding a layer of looser filamentous hyphae and an inner core of prosenchymatous cells (Fig. 2a). The filamentous hyphae were thick-walled and $2.5\text{ }\mu\text{m}$ in diameter with simple septa and a narrow lumen (Fig. 2b); clamp connections were present. Prosenchymatous cells were rounded to irregular in shape with very thick walls and a narrow lumen (Fig. 2c). No fruiting structures were observed. The structure of the fungal material was typical of a sclerotium³ and we concluded that the club-heads were basidiomycetous sclerotia. Samples of tissues were sent to the Forest Research Station, Bulolo, where the fungus was identified as conspecific with *Lentinus tuber-regium* (Fr.) Fr.

There is a lack of stone in the area where the Gogodala live and the sclerotia seem to be used as a substitute. Throughout the vast alluvial swampland of south-central New Guinea the indigenous peoples commonly substitute wood for stone in the manufacture of club-heads⁴. But as far as we have been able to determine this is the first recorded use of a fungus for such purposes in Papua New Guinea; neither is there any record of fungi being used for such purposes in other parts of the world. Today, after a generation of acculturation, the primary use of these club-heads is to adorn clubs carried during dance activities, although clubs with fungal club-heads attached are some-

times taken on hunting expeditions and, according to older informants, were also once carried into war.

We thank Dr P. H. B. Talbot for comments on the microscopical preparations. J.A.B. was supported by the National Geographic Society.

T. V. PRICE*

Department of Biology,
University of Papua New Guinea

J. A. BALDWIN

Department of Geography,
Bowling Green State University,
Ohio 43403

J. A. SIMPSON

Forest Research Station,
PO Box 134,
Bulolo, Papua New Guinea

Received 4 January; accepted 6 April 1978.

*Present address: Biology Department, Hawkesbury Agricultural College, New South Wales 2753, Australia.

1. Massal, E. & Barrau, J. *South Pacific Commission Tech. Paper* 94 (1956).
2. Heim, R. & Wasson, R. G. *Harvard University Botanical Museum Leaflets* 21, 1-36 (1965).
3. Talbot, P. H. B. *Principles of Fungal Taxonomy* (Macmillan, London, 1971).
4. Speiser, F. *Zeitschrift für Ethnologie* 64, 74-105 (1932).

An explanation of ecological and developmental constants

HUTCHINSON¹ noted that congeneric species which diverge in size when sympatric tend to differ in the length of their feeding structures by a factor of about 1.28. He postulated that this average amount of character displacement represented the lower limit to similarity for competing species. It has also been noted that the relative size increase between larval instars of many arthropods (Dyar's constant)², or between year classes in many salamanders (as reported here), has the same magnitude as Hutchinson's constant. The similarity of this developmental constant to the ecological constant suggests that the degree of divergence between age classes has the same ecological basis as that between competing species. Although many examples of these apparent ecological and developmental constants have been documented, there is yet no mechanism to explain either their relative degree of constancy or their average magnitude³. Here I offer a possible explanation for both.

I postulate that size-displaced species or age classes within a species diverge by a relatively constant percentage, regardless of their absolute body size, because they tend to show a relatively constant amount of variability in morphology. If ecological separation by size requires a certain minimum limit to overlap in the frequency distributions of two species or age classes, and if they tend to show the same degree of morphological variability, then their proportionate divergence in mean size will also be relatively constant. Simpson *et al.*⁴ observed that the coefficient of variations (CV) for many structures tends to range between 4 and 10, most frequently between 5 and 6. For two species in which the feeding structure of the larger is 1.28 times the size of that of the smaller, a CV of 5.5 gives an overlap in their frequency distributions of 1 or 2%.

This explanation for the relative constancy of size divergence between competing species or age classes also predicts that the 'constant' should be somewhat variable, because the amount of morphological variability shown by species is not absolutely constant. More specifically, the magnitude of separation of two size-displaced species or age classes is expected to be directly proportional to their morphological variability. Several observations on the amount of size divergence among character-displaced sympatric species and among immature age classes of arthropods and salamanders support this prediction (Fig. 1). Structures with

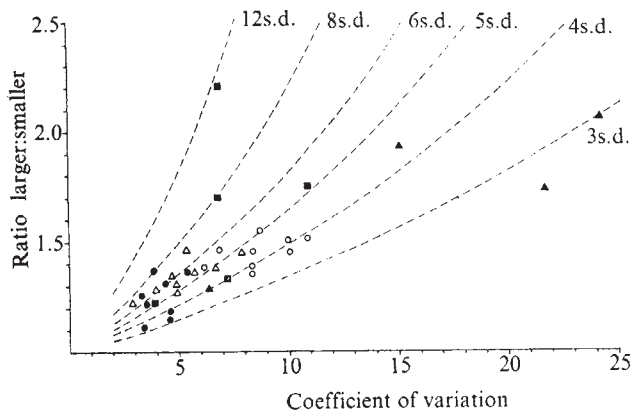


Fig. 1 The amount of size displacement between populations as a function of morphological variability for linear measurements. The amount of size displacement is measured by the ratio of the larger species or size class divided by the smaller. The lines are calculated values of the ratio when the means of logarithmically transformed normal distributions are separated by the designated number of standard deviations (s.d.). Each point is the average of the means for all sympatric species in a genus (or less often in a feeding guild) or all size classes within a species. Closed symbols (refs 17–28 and my unpublished data) represent sets of congeneric species (or otherwise related species) for which the original paper postulated the size differences to be related to their co-existence. Only adults were used in the analysis. Triangles represent arthropods; circles, vertebrate homeotherms; squares, vertebrate poikilotherms. Open symbols (refs 29–44 and my unpublished data) represent larval instars of arthropods (triangles) and year classes of salamanders (circles). Only studies in which the animals were collected in the field or reared in semi-natural conditions were used. Animals reared in the laboratory gave appreciably less overlap.

very high *CV* values are unlikely to be subject to much stabilising selection and are therefore unlikely to be critically involved in competitive coexistence. The only point in Fig. 1 with less separation than 3 standard deviations was for ovipositors of three species of parasitic wasps which differed in the degree of decay of logs into which they probed. The ovipositor differences were probably secondary to this behavioural difference.

More indirectly, Selander⁵ noted that families of birds showing the greatest amount of sexual dimorphism implying greater population variance than monomorphic species, were the same families for which Schoener⁶ had found sympatric congeners to be most divergent in bill size. The observed relationship between variation and size divergence (Fig. 1) indicates that the degree of overlap in the frequency distributions of two populations is more basic than the magnitude of the divergence.

Figure 1 suggests that the magnitude of size displacement between two populations lies between 3 and 8 standard deviations of the logarithmically transformed normal distributions, making it 3 to 8 times greater than the minimum displacement postulated for resource use⁷. An explanation for this difference is that morphological variation is always considerably less than variation in use of a resource. Complete separation in morphology may generally be necessary to achieve sufficient separation in resource use (Fig. 2).

A few examples drawn from terrestrial animals support the supposition that resource use is more variable than morphology (Table 1). Variability in resource use ranges from 3 to 16 times greater than variability in morphology. If mean resource item size increases on a logarithmic scale at the same rate as the mean change in morphology, then the observed divergence in size of the feeding structures is generally consistent with the postulated displacement in mean resource item size of about 1 standard deviation of the resource distribution. No strong conclusion, however, can be drawn from this comparison because the amount of overlap that can be tolerated for coexistence depends on

whether or not the resource in question is the only one involved. Furthermore, meaningful comparisons can be made only on the resource(s) which regulate the densities of the populations and on the structures involved in the use of such resources^{8,9}.

Displacement in size may not always be related to use of size-dependent resources. Instead it could be related to the ability to interfere with the resource use of the other population. One species (or age class) may get sufficiently larger than the other in order to oust it from a preferred habitat, feeding perch, or other resource. In such a case all individuals of the larger population may have to be larger than the largest individual of the smaller or else they would be at a disadvantage in interference.

I have argued that size-displaced species show a relatively constant amount of overlap in their distributions, the exact amount of displacement being a function of the variability of the populations under consideration. This implies that variability influences displacement, but the amount of displacement may also feed back to influence variability. Thus in some habitats a large number of competitors or the size range of a resource may reduce the possible amount of size displacement among species and hence select for lower variability within a population. Alternatively, ecological opportunity may often favour an increase in variability of a species¹⁰.

In conclusion, the observations presented here suggest that there is a tolerable overlap in linear measurements of 0 to 5% (rarely 10%) between competing species (and perhaps age classes) which diverge in size. There is no simple explanation for the observed amount of overlap, for morphological divergence may be related to resource use in various ways. Also, selection to minimise overlap in natural communities may be balanced by invasion by new species or by constraints on growth rate and time of oviposition. However, the demonstration that the ecological and developmental constants are probably real provides a firm base from which to investigate the nature of competitive interactions in natural communities.

Horn and May³ have noted that the 'Dyar-Hutchinson rule' holds for instars of children's bicycles, for sets of kitchen skillets, for ensembles of recorders and for consorts of viols. They concluded that the rule may have more to do with assembling sets of tools than with anything directly biological. Although I have additionally found that the rule holds for sets of plates, glasses and small pewter cans, I have also found that it holds for sets of animal figurines. Each set of figurines measured contained three animals in a graded sequence of size. Six such sets gave an average ratio of 1.29 with a range from 1.19 to 1.41. Two friends,

Fig. 2 Diagram illustrating the amount of overlap in feeding structures (a) and in resource use (b). The variability of resource use is four times greater than that of feeding structures but mean size of the resource is proportional to mean size of the feeding structure. That is, feeding structures and resources each have the same size ratio.

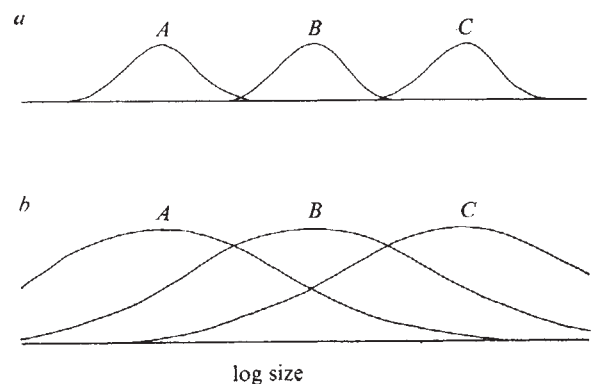


Table 1 Relationship between coefficients of variation for feeding structures and for various size-dependent resources

Taxon	Structure	CV	Resource	CV	Ratio of CVs	Source
<i>Batrachoseps</i> , <i>Aneides</i>	Head width	5.8	Salamanders { Prey length Prey width	69.7 77.5	12.0 13.4	*
<i>Plethodon</i>	Head width	12.6	Prey width	80.7	6.4	*
<i>Plethodon</i>	Head width	10.6	Shelter size	28.3	2.7	Ref. 11
<i>Sceloporus</i>	Head length	23.0	Lizards Prey length	60.5	2.6	Ref. 12
<i>Anolis</i>	Head length	14.5	Prey length	49.1	3.4	Ref. 13
<i>Anolis</i>	Head length	7.7	{ Prey length Branch diameter	68.4 70.2	8.9 9.1	Ref. 14
Flycatchers	Bill length	3.8	Birds Prey length	61.8	16.3	Ref. 15
Sparrows	Bill length	5.5	Seed length and width	18.8	3.4	Ref. 16

The data were either taken directly or calculated from information available in the source listed. All values are averages from several species and refer to single size groups.

*My unpublished data.

one by request and the other not, made sets of four to six size-displaced objects, which gave an average ratio of 1.31 with a range from 1.14 to 1.59. An explanation for these observations may have to do with our perceptual abilities, a cause that may also be applicable to (and perhaps derived from) the natural world.

I thank Valerie Maiorana and Leigh Van Valen for their help. This research was supported by a US NIMH post-doctoral fellowship.

VIRGINIA C. MAIORANA

Department of Biology,
University of Chicago,
Chicago, Illinois 60637

Received 13 February; accepted 6 April 1978.

- Hutchinson, G. E. *Am. Nat.* **93**, 145-159 (1959).
- Enders, F. *Environ. Ent.* **5**, 1-10 (1976).
- Horn, H. S. & May, R. M. *Nature* **270**, 660-661 (1977).
- Simpson, G. G., Roe, A. & Lewontin, R. C. *Quantitative Zoology*, 2nd edn (Harcourt, Brace and World, New York, 1960).
- Selander, R. K. *Condor* **68**, 113-151 (1966).
- Schoener, T. W. *Evolution* **19**, 189-213 (1965).
- May, R. M. & MacArthur, R. H. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1109-1113 (1972).
- Van Valen, L. *Evol. Theory* **1**, 31-49 (1973).
- Maiorana, V. C. *Can. J. Zool.* (in the press).
- Van Valen, L. *Am. Nat.* **99**, 377-391 (1965).
- Fraser, D. F. *Ecology*, **57**, 238-251 (1976).
- Rose, B. R. *Ecology*, **57**, 531-541 (1976).
- Roughgarden, J. *Am. Nat.* **108**, 429-443 (1974).
- Schoener, T. W. *Ecology* **49**, 704-726 (1968).
- Root, R. B. *Ecol. Monogr.* **37**, 317-350 (1967).
- Allaire, P. N. & Fisher, C. D. *Auk* **92**, 260-269 (1975).
- Lack, D. *Darwin's Finches* (Cambridge University Press, Cambridge, 1947).
- Cranbrook, Lord *Proc. zool. Soc., Lond.* **128**, 597-600 (1957).
- Grant, P. R. *Evol. Biol.* **8**, 237-337 (1975).
- Grant, P. R. *Postilla* **90**, 1-106 (1965).
- Grant, P. R. *Syst. Zool.* **21**, 289-291 (1972).
- Huey, R. B., Pianka, E. R., Egan, M. E. & Coons, L. W. *Ecology* **55**, 304-316 (1974).
- Schoener, T. W. & Gorman, G. C. *Ecology* **49**, 819-830 (1968).
- McEachran, J. D. & Martin, C. O. *Environ. Biol. Fish.* **2**, 121-130 (1977).
- Heatwole, H. & Davis, D. M. *Ecology* **46**, 140-150 (1965).
- Maly, E. J. & Maly, M. P. *Oecologia* **17**, 325-333 (1974).
- Price, P. W. *Ecology* **53**, 190-195 (1972).
- Culver, D. C. *Ecology*, **51**, 949-958 (1970).
- Walt, J. F. & McPherson, J. E. *Ann. ent. Soc. Am.* **66**, 1103-1107 (1973).
- Van Derwerker, G. K. & Kulman, H. M. *Ann. ent. Soc. Am.* **67**, 29-31 (1974).
- Hoxie, R. P. & Wellso, S. G. *Ann. ent. Soc. Am.* **67**, 183-186 (1974).
- Hatchett, J. H., Daugherty, D. M., Robbins, J. C., Barry, R. M. & Houser, E. C. *Ann. ent. Soc. Am.* **68**, 209-213 (1975).
- Nielson, M. W., May, C. J. & Tingly, W. M. *Ann. ent. Soc. Am.* **68**, 401-403 (1975).
- Jennings, D. T. *Ann. ent. Soc. Am.* **68**, 597-606 (1975).
- Kirkland, R. L. & Goeden, R. D. *Ann. ent. Soc. Am.* **70**, 583-587 (1977).
- Reyment, R. & Van Valen, L. *Bull. geol. Inst. Univ. Upsala N. S.* **1**, 83-94 (1969).
- Tilley, S. G. *Ecology*, **54**, 3-17 (1973).
- Houck, L. D. *Copeia* **1977**, 70-83 (1977).
- Peacock, R. L. & Nussbaum, R. A. *J. Herpetol.* **7**, 215-224 (1973).
- Bruce, R. C. *Copeia* **1971**, 234-246 (1971).
- Bruce, R. C. *J. Herpetol.* **6**, 43-51 (1972).
- Bruce, R. C. *Copeia* **1975**, 129-137 (1975).
- Sayler, A. *Copeia* **1966**, 183-193 (1966).
- Highton, R. *Copeia* **1962**, 597-613 (1962).

Very low calcium content of cochlear endolymph, an extracellular fluid

IONIC calcium is important in many membrane functions and the possibility of it being involved in the cochlear transduction processes is supported by the scanty evidence available¹. In the analogous lateral line organ of sub-mammalian species, the mechanosensitivity of the hair cells is related directly to the external calcium concentration at their specialised receptor poles². There is also evidence of interaction with monovalent cation effects^{2,3}. In mammals, the cochlear duct is filled with endolymph, the unusual constitution of which (in the rat d.c. potential + 88 mV, K⁺ 148 mM, Na⁺ 0.84 mM) is seemingly maintained entirely by active transport mechanisms⁴ and is generally conceded to be necessary for normal transduction. We report here that the cochlear endolymph calcium occurs almost entirely in the non-complexed state at a concentration of 3×10^{-5} M in the rat. This is substantially below the usual extracellular concentration, and must influence the function of the receptor pole membranes and might well affect the role of calcium in the transduction process in the cochlea. The total magnesium concentration we measured was 1×10^{-5} M.

Earlier attempts at analysis of utricular endolymph have given total calcium concentrations of 1.5×10^{-3} M (ref. 5) and 4.5×10^{-4} M (ref. 6), with a total magnesium concentration of 5×10^{-4} M (ref. 5). These values are less than those in serum, even though overestimation from contamination is now known to have been likely with the techniques used. The concentration of sodium in the utricular endolymph is more than ten times greater than that in the cochlea⁷, which prevents extrapolation between the two fluids but which admits the possibility of a still lower cochlear calcium concentration. We investigated this concentration directly by two independent methods.

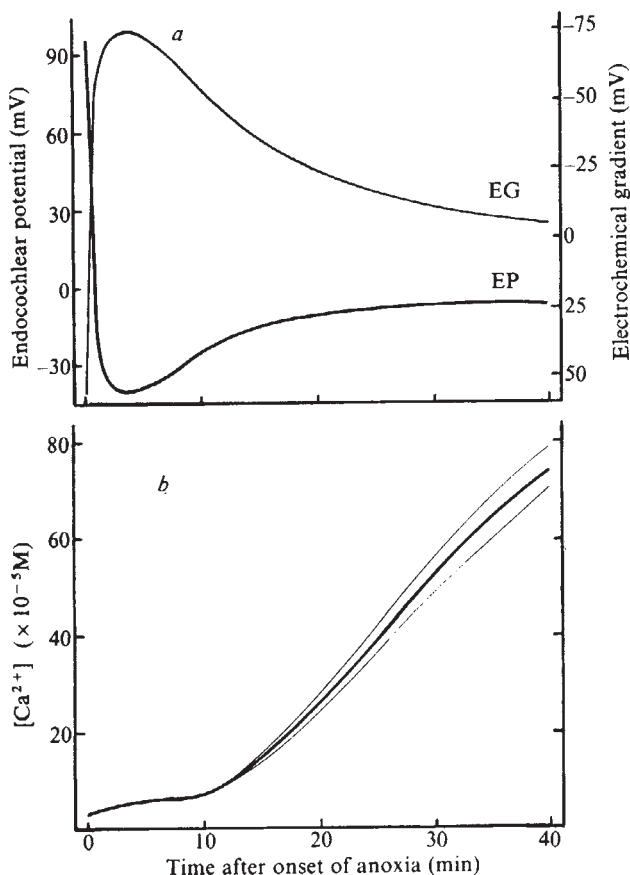
Inbred Sprague-Dawley descended albino rats (150-200 g) were anaesthetised with sodium pentobarbitone, the endocochlear d.c. potential was measured by introduction of a conventional microelectrode into the middle turn of the left cochlea, up to 150 nl of endolymph was removed using an aspirating pipette, and the sodium content of the aspirated fluid was determined using total-emission flame spectrophotometry⁸. Total calcium and magnesium were estimated separately by integration of the emission in an oxy-hydrogen flame at 422.7 or 285.2 nm. The original instrument was modified for this purpose, sample aliquots (about 2×10^{-11} g calcium and 3×10^{-12} g magnesium) being introduced into the hydrogen supply of the flame by vaporisation from an electrically heated tungsten filament.

The total calcium concentration in the endolymph was 2.3×10^{-5} M (s.e. $\pm 0.3 \times 10^{-5}$; $n=5$) and the total magnesium

concentration was 1.1×10^{-5} M ($\pm 0.2 \times 10^{-5}$; $n=5$). The endocochlear potentials (mean 86.9 mV) and the endolymph sodium concentrations (mean 0.80 mM) were normal. Calcium contamination of the samples during handling was a serious problem and concurrent estimation of distilled water control samples (3×10^{-7} M Ca; 7×10^{-7} M Mg) was essential.

In further experiments, a calcium-sensitive double-barrelled microelectrode was inserted into the left middle turn. The electrodes were as described by Oehme *et al.* with a linear response to at least 1×10^{-5} M CaCl_2 . Calibration was performed at the animal temperature (35°C) in solutions simulating endolymph and of constant osmolarity. The second barrel of the electrode was used to measure the endocochlear potential, which was normal in all cases (mean 90.2 mV). The concentration of ionised calcium in the endolymph was 3.0×10^{-5} M ($\pm 0.2 \times 10^{-5}$; $n=15$). This is not significantly different from the value obtained in the aspiration experiments as judged by Student's two-tailed *t* test ($0.1 < P < 0.2$), revealing that the calcium exists almost entirely in the simple (non-complexed) form.

Fig. 1 Changes in endolymph during anoxia produced by the intravenous injection of 2 ml of air into the right femoral vein. *a*, Mean endocochlear potential in three experiments (EP) and calculated average electrochemical gradient between endolymph and perilymph for Ca^{2+} (EG). The s.e.m. of the endocochlear potential was ± 3 – ± 1 mV. A negative electrochemical potential indicates a driving force into endolymph. The rapid reversal of the electrical potential difference produced a corresponding reversal of the ionic electrochemical gradient. *b*, Calcium concentration, showing the mean (heavy line) and s.e.m. (lighter lines) in the three experiments shown in (*a*). The increased rate of entry after 9 min occurred in spite of a progressive decrease in the driving force for Ca^{2+} .



Subsequently, the perilymph calcium concentrations were measured in the left basal turn and, in this instance, the microelectrode was calibrated in solutions simulating perilymph. The concentration of ionised calcium in scala vestibuli was 0.64×10^{-3} M $\pm 0.05 \times 10^{-3}$ ($n=6$) and in scala tympani 0.68×10^{-3} M $\pm 0.04 \times 10^{-3}$ ($n=6$). A two-tailed *t* test established that there was no significant difference between these two values ($0.5 < P < 0.6$). This is in contrast to the report⁹ of small differences in the sodium and potassium concentrations, although these have not been confirmed¹⁰.

Finally, the changes produced by anoxia in the endolymph concentration of Ca^{2+} were followed in a further three animals. The microelectrode selectivity was not affected by the simultaneous pH alterations. The average endocochlear potential fell from 95.7 mV to a minimum of -39.7 mV at 3.8 min, a typical finding. The calcium concentration increased progressively in a roughly biphasic fashion (Fig. 1), with a substantial increase in the rate of entry at about 9 min. A qualitatively similar increase in the rates of change in the sodium and potassium concentrations has been reported at 14 min (ref. 11). The possible relationship between these phenomena remains to be examined. Almost certainly, the alterations in calcium concentration result partly from the movement of calcium across the boundary membranes. In addition, complexed calcium might be released relatively rapidly from the tectorial membrane, which largely fills the cochlear duct, possibly by the effect of the fall in pH on hydrogen bonding.

Although the concentration of calcium in the rat endolymph (3×10^{-5} M) is unusually low for an extracellular fluid, it is considerably greater than the equilibrium concentration (7×10^{-7} M) calculated using the Nernst equation and the measured perilymph level. Homeostasis must be maintained, consequently, by mechanisms transporting calcium into the endolymph. Anoxic inhibition of these mechanisms alone would produce a decreased endolymph concentration but this is counteracted largely by the concomitant reversal of the electrical gradient (Fig. 1). The reason for the low normal level and its influence on hair cell transduction are speculative; investigation is continuing.

We thank Professor W. Simon for the neutral carrier calcium ion-exchanger, and the Smith Kline and French Foundation for financing the flamephotometer modifications.

S. K. BOSHER

*Ferens Institute of Otolaryngology,
Middlesex Hospital Annexe,
Cleveland Street,
London W1, UK*

R. L. WARREN

*Department of Nuclear Medicine,
Thorn Institute,
Middlesex Hospital Medical School,
London W1, UK*

Received 3 March; accepted 12 April 1978.

- Schacht, J. *J. acoust. Soc. Am.* **59**, 940–944 (1976).
- Sand, O. *J. comp. Physiol.* **102**, 27–42 (1975).
- Yoshioka, T., Kawai, K. & Katsuki, Y. *Jap. J. Physiol.* **26**, 441–453 (1976).
- Bosher, S. K. & Warren, R. L. *Proc. R. Soc. B171*, 227–247 (1968).
- Citron, L. & Exley, D. *Proc. R. Soc. Med.* **50**, 697–701 (1957).
- Rauch, S. & Rauch, I. in *Handbook of Sensory Physiology V/1* (eds Keidel, W. D. & Neff, W. D.) 661 (Springer, Berlin, 1974).
- Sellick, P. M., Johnstone, J. R. & Johnstone, B. M. *Pflügers Arch. ges. Physiol.* **336**, 21–27 (1972).
- Oehme, M., Kessler, M. & Simon, W. *Chimia* **30**, 204–206 (1976).
- Silverstein, H. & Takeda, T. *Ann. Otol. Rhinol. Laryngol.* **86**, 493–499 (1977).
- Melichar, I. & Syka, J. *Pflügers Arch. ges. Physiol.* **372**, 207–213 (1977).
- Bosher, S. K. *J. Physiol., Lond.* **266**, 93–94P (1977).

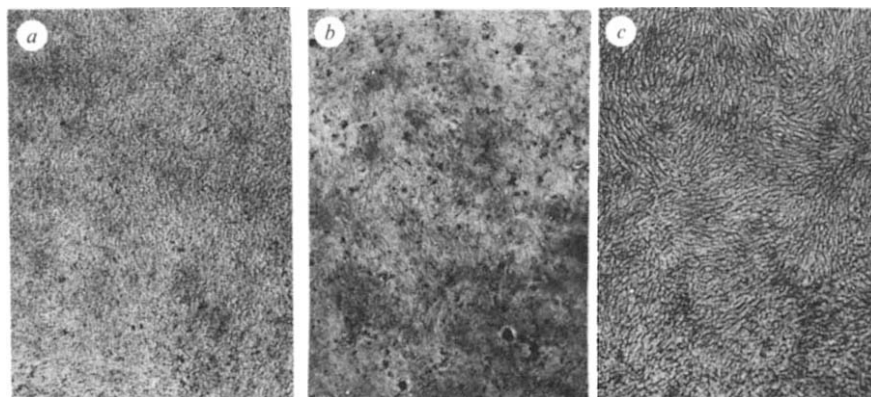
Retinol induces density-dependent growth inhibition and changes in glycolipids and LETS

CHEMICAL carcinogenesis and viral transformation are prevented or inhibited by vitamin A or its analogues^{1,2}. Vitamin A may also play a part in membrane glycosylation as a lipid intermediate^{3,4}. Recently, effects of retinoic acid on proliferation and density-dependent growth of mouse L cells⁵ and many other untransformed and transformed cells *in vitro* have been described⁶. In view of these observations, we have studied the effect

cultures on addition of vitamin A. This pattern is reminiscent of normal NIL cell cultures with low passage number, which also show low tumorigenicity. Debris associated with overgrowth have been observed (see Fig. 1 for NILpy cells). NIL cells showed essentially the same morphological changes as NILpy cells on addition of retinol, but to a lesser degree.

A remarkable change in the morphology of 3T3 cell was observed during culture with retinol and retinoic acid; that is, the 'cobble stone' appearance was not conspicuous at confluency, the cells appeared elongated and well orientated. NILpy cells in irradiated medium showed a slightly reduced growth rate, but the addition of retinol induced a remarkable density-dependent inhibition of cell growth (see Fig. 2a) in parallel to the change of morphology as shown in Fig. 1. NIL cells in

Fig. 1 Effect of retinol on morphology of NILpy cells at confluency. Hamster NILpy cells^{7,8} were grown in Dulbecco modified Eagle's medium supplemented with *a*, 10% foetal calf serum (regular medium); *b*, ultraviolet light-irradiated foetal calf serum (vitamin A free medium), and *c*, irradiated foetal calf serum supplemented with 20 nmol ml⁻¹ retinol (retinol added medium). All culture media were supplemented with penicillin and streptomycin. Foetal calf serum was irradiated with a 'Pen Ray' ultraviolet lamp (Ultraviolet Product, Inc.) by insertion of the lamp in serum and irradiation for 15 min. During the course of cell growth, morphology was observed and occasional pictures taken under a phase-contrast microscope.



of retinol on cell growth behaviour, glycolipid synthesis, and surface-exposed profile of glycoproteins in hamster fibroblasts NIL, NILpy and mouse (BALB/c) 3T3 cells. We report here that culturing NIL or 3T3 cells in vitamin A-containing medium markedly enhances, whereas a medium with ultraviolet-irradiated serum markedly reduces, contact orientation and cell-density dependent inhibition of cell growth. Associated changes of cell surface membrane GM₃ level, ganglioside contact response, and in LETS ('Gap a') were observed.

A remarkable change in the morphology of NILpy cells was caused by addition of 20 nmol of retinol per ml of medium. The irradiated medium lacking vitamin A caused a less clear cell boundary and the cells became flattened. 'Contact orientation' and parallel growth pattern were induced in NILpy cell

irradiated medium did not grow well, addition of retinol 20 nmol ml⁻¹ restored growth to the same level as in regular medium. In the presence of higher concentrations of retinol (100 nmol ml⁻¹) a density-dependent inhibition was observed in parallel to the appearance of contact orientation (data not shown). A similar density-dependent inhibition of cell growth and reduced saturation density was observed in 3T3 cells grown in retinol (Fig. 2b). A dose-dependent response of the reduction in growth rate was observed. Retinoic acid caused a greater reduction in saturation density (Fig. 2b).

The change in growth behaviour induced by the absence or the presence of retinol was associated with an effect on glycolipid synthesis. Among the glycolipid changes caused by retinol, the haematoid level was the most remarkable. Haematoid

Table 1 Effect of retinol on glycolipid synthesis in NIL2K and NILpyT cells

	NIL2K				NILpyT		
	Regular	Irradiated	Irradiated + retinol (20 nmol ml ⁻¹)	Irradiated + retinol (100 nmol ml ⁻¹)	Regular	Irradiated	Irradiated + retinol (20 nmol ml ⁻¹)
GM ³ (5 h)	6,900	1,650	2,930	8,520			
(15 h)	5,800	2,200	1,900	7,580			
(24 h)	9,800	3,100	3,200	10,500	10,325	9,564	16,397
Ceramide monohexoside	12,021	16,699	13,210	11,230	11,823	10,726	9,947
Ceramide dihexoside	11,500	8,500	7,410	7,250	2,241	2,848	2,599
Ceramide trihexoside	5,860	9,600	8,900	9,200	3,092	3,879	2,852
Ceramide tetrahexoside	5,650	10,120	7,500	6,500	3,255	4,411	2,556
Forssman glycolipid	10,130	10,490	10,995	11,500	6,148	6,969	4,268

Values indicate activities of each glycolipid in c.p.m. per 10 mg of cell residues. Except where indicated, values were obtained from cells incubated for 24 h with ¹⁴C-galactose. Glycolipid synthesis was studied with cells cultured in the presence of uniformly labelled ¹⁴C-galactose. Approximately 2–3 × 10⁴ cells cm⁻² were seeded in various media (8 cm diameter Petri dishes) and incubated with various media for 5–7 d. Morphological changes were observed under the phase contrast microscope. The confluent cell cultures were washed twice with phosphate-buffered saline (PBS) pH 7.4 and then added with three kinds of medium as described above—regular (R), irradiated (I) and vitamin A added (I+A). The volume of fresh medium was 3 ml and it contained 1.5 μCi of uniformly labelled ¹⁴C-galactose. The cells were incubated for 5, 15 and 24 h at 37 °C. Cells were then collected with a rubber policeman and washed with PBS pH 7.4 and the cell pellets were stored at -70 °C until used. The labelled cells were extracted with chloroform-methanol 2:1 in a Sorval Omnimixer for 2 min in an ice bath. Extracts were filtered through glass filter paper which was preweighed and washed twice with chloroform-methanol 2:1. The solid residues were dried and weighed. The extracts were evaporated and mixed with cold glycolipid mixture. The total glycolipid was isolated as acetate according to the method of Saito and Hakomori¹⁴. Glycolipids were further fractionated into neutral and acidic glycolipid (ganglioside) fractions with DEAE-Sephadex using the method of Yu and Ledeen¹⁵. The neutral and acidic glycolipids were analysed separately on thin-layer chromatography in solvent chloroform-methanol-water 60:35:8. The chromatogram was recorded by autoradiogram and each spot of glycolipid was scraped from thin-layer plate and counted in a scintillation counter with 0.5 ml of NCS solubiliser and 10 ml of toluene-based scintillation counting fluid.

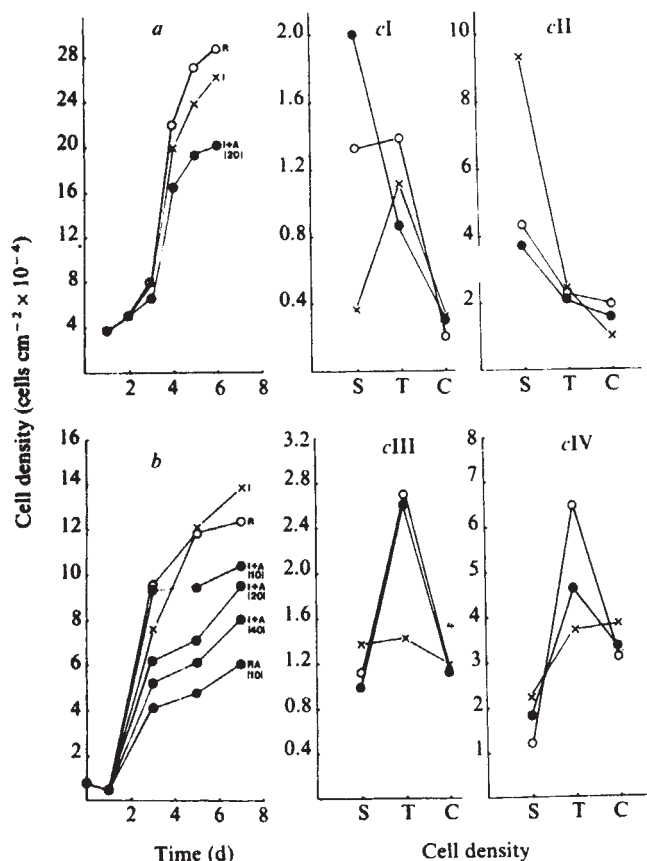


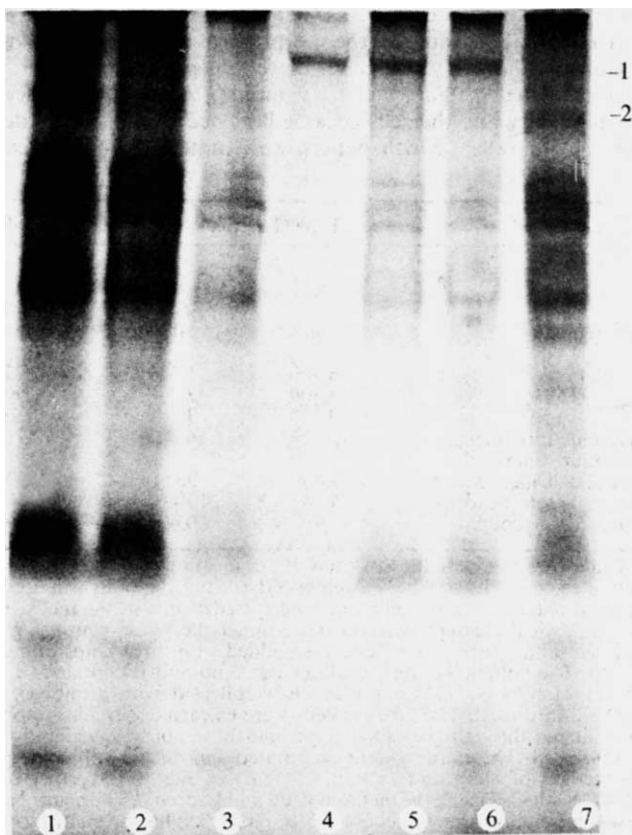
Fig. 2 Effect of retinol on cell growth and contact-dependent ganglioside synthesis. *a*, NIL cell growth curve; *b*, 3T3 cell growth curve. The growth curves in different media are indicated as follows: R, regular medium; I, irradiated, retinol-free medium; I+A, irradiated and vitamin A (retinol) added medium; RA, retinoic acid added medium. Numbers (present under I+A) indicate the quantity of retinol (nmol ml^{-1}) added in the medium. *c*, The response of ganglioside synthesis at different degrees of cell contact and the effect of retinol on the ganglioside response. BALB/c 3T3 cells were grown in different population densities, sparse, touching, and crowded status according to the conditions previously described⁹, that is, $1-2 \times 10^3$ cells cm^{-2} seeded and collected after 14–20 h for sparse culture; $1-2 \times 10^4$ cells cm^{-2} seeded and harvested after 30–48 h for a 'touching' culture. The same number of cells seeded and collected after 50–58 h obtained a 'crowded' culture. Cell density was determined by examination of the cell layers with a phase contrast microscope. Aliquots of the cell suspensions after labelling were taken for analysis of protein by the method of Lowry¹⁷. Sparse, touching, and crowded cultures contained 0.04–0.05 mg per plate, 0.4–0.6 mg per plate, and 1.4–1.5 mg per plate protein, respectively. These cells with differing degrees of cell contact were cultured in Dulbecco modified Eagle's medium with regular, ultraviolet-irradiated, and retinol added condition as described in Fig. 1. These cultures were metabolically labelled in the presence of uniformly labelled ^{14}C -galactose or ^{14}C -glucosamine. Cells were collected, washed, extracted, and glycolipids were analysed as for Table 1. The activity of the individual bands corresponding to GM_3 (*cI*), GM_2 (*cII*), GM_1 (*cIII*), and GD_{1a} (*cIV*) were scraped from thin-layer plates and counted in a scintillation counter. The ordinate indicates activities of each ganglioside in c.p.m. per mg of cell protein. The abscissa indicates cell population density, S, T, and C, stand for, respectively, sparse, touching, and crowded cell cultures. $\times$, Culture with ultraviolet-irradiated serum $\circ$, Culture with regular serum (indicated as R). $\bullet$, Culture with vitamin A (20 nmol ml^{-1}) added serum.

synthesis in NIL2K cells was greatly reduced in irradiated medium, and was restored or enhanced on addition of retinol. Haematoside synthesis in NILpyT cells was only slightly reduced in the irradiated medium, but was greatly enhanced on addition of retinol. This response of haematoside synthesis is parallel to the growth inhibition induced by retinol.

The pattern of ganglioside synthesis in BALB/c 3T3 cells depends greatly on the state of cell contact⁹, therefore the synthesis in 3T3 cells at various cell population densities was studied in the presence and absence of vitamin A. As seen in Fig. 2c, GM_3 synthesis is much higher in the presence of vitamin A in sparse cell culture, similarly GM_1 and GD_{1a} synthesis is higher in the presence of retinol at touching phase. This indicates that retinol greatly promotes cell contact-dependent enhancement of GM_1 and GD_{1a} synthesis.

In contrast to a striking difference in haematoside synthesis in the presence or absence of retinol, the surface-exposed glycoprotein showed relatively little change in NILpyT cells. However, in NIL2K cells retinol induced a significant change in the surface-labelled glycoproteins, namely, 'galactoprotein a (Gap a)' or 'LETS'¹⁰⁻¹³ decreased significantly and the label in a new band with a relative molecular weight 180,000 was increased. The change in growth behaviour induced by retinol may be correlated with the change in haematoside synthesis in NIL and NILpyT cells, the change in 3T3 cells may well be

Fig. 3 Surface-labelled glycoprotein patterns of NIL cells by the neuraminidase-galactose oxidase and ^{35}S -methionine sulphone hydrazide method. NILpy cells or NIL2K cells were grown in regular, ultraviolet-irradiated, or vitamin A added media as described in Fig. 1. The profile of cell surface glycoproteins was revealed by treating cells with neuraminidase, galactose oxidase, and ^{35}S -methionine sulphone hydrazide as previously described¹⁶. Lanes 1, 2 and 3 are NILpyT cells cultured in regular, ultraviolet-irradiated, and retinol-added media, respectively. Lanes 5, 6 and 7 are patterns of NIL2K cells grown in regular, ultraviolet-irradiated, and retinol-added media, respectively. Lane 4 is the pattern of NIL2K cells grown in regular medium and labelled without neuraminidase treatment. Band 1 indicates the position of LETS (Gap a) and Band 2 (with a relative molecular weight 180,000) indicates the position of the new band which is heavily labelled in cells grown in the presence of retinol.



correlated to the increase in ganglioside synthesis upon cell contact. This response was significantly enhanced in 3T3 cells cultured in the presence of retinol and decreased in the absence of retinol. The mechanism of the contact response of ganglioside synthesis is not known, but vitamin A compounds may be essential to maintain some unknown membrane mediated growth control involving glycolipids or glycoproteins. Recently, the role of a cell surface protein, Gap a or LETS, in intercellular adhesion and cell growth control has received a great deal of interest¹⁰⁻¹³. The vitamin A compounds induced disappearance of this glycoprotein in NIL2K cells is significant, although the change itself is in the opposite direction. Possibly, the disappearance of LETS is supplemented by the appearance of a new surface protein which may be functionally more potent than LETS.

The effect of vitamin A compounds to prevent carcinogenesis^{1,2} could be in some way related to the changes in surface membrane glycolipids and glycoproteins described here. On the other hand, glycosyltransferase level for the synthesis of haematoside (CMP-sialic acid: lactosylceramide sialyltransferase) of NIL cells grown in medium with added retinol did not significantly differ from that of cells grown in normal medium. Therefore, the biochemical basis of the observed effect on glycolipid synthesis and surface-labelled glycoproteins could be a more complex matter and most probably the change can be caused by the level of membrane organisation.

This work was supported by US NCI grant CA20026. L.M.P. received University Pathobiology Training Grant 1 T32 GM07187.

LEONARD M. PATT
KOICHI ITAYA*
SEN-ITIROH HAKOMORI

Biochemical Oncology, Fred Hutchinson
Cancer Research Center, and
University of Washington,
Seattle, Washington 98104

Received 23 January; accepted 29 March 1978.

* Permanent address: Department of Physiological Chemistry, Hokkaido University, Sapporo, Japan.

- Sporn, M. B., Dunlop, N. M., Newton, D. L. & Smith, J. M. *Fedn Proc.* 35, 1332-1338 (1976).
- Seifter, E., Zisblatt, M. & Levine, N. *Life Sci.* 13, 945 (1973).
- Fort-Coutaz, J. P., Silverman-Jones, C. S. & DeLuca, L. M. *J. Lipid Res.* 17, 220-230 (1976).
- DeLuca, L. M., Fort-Coutaz, J. P., Silverman-Jones, C. S. & Roller, P. R. *J. Biol. Chem.* 252, 2575-2657 (1977).
- Dion, L. D., Blalock, J. E. & Gifford, G. E. *J. natn. Cancer Inst.* 58, 795-801 (1977).
- Lotan, R. & Nicolson, G. L. *J. natn. Cancer Inst.* 59, 1717-1722 (1977).
- Diamond, L. *Int. J. Cancer* 2, 143-152 (1967).
- Gahmberg, C. G. & Hakomori, S. *J. Biol. Chem.* 250, 2438-2446 (1975).
- Yogeeswaran, G. & Hakomori, S. *Biochemistry* 14, 2151-2156 (1975).
- Gahmberg, C. G. & Hakomori, S. *Proc. natn. Acad. Sci. U.S.A.* 70, 3329-3333 (1973).
- Hynes, R. O. *Proc. natn. Acad. Sci. U.S.A.* 70, 3170-3174 (1974).
- Vaheri, A. & Ruoslahti, E. *Int. J. Cancer* 13, 579-586 (1974).
- Hynes, R. O. *Biochim. biophys. Acta* 458, 73-107 (1976).
- Saito, T. & Hakomori, S. *J. Lipid Res.* 12, 257-259 (1971).
- Yu, R. K. & Ledeen, R. W. *J. Lipid Res.* 13, 680-686 (1972).
- Itaya, K., Gahmberg, C. G. & Hakomori, S. *Biochem. biophys. Res. Commun.* 64, 1028-1035 (1975).
- Lowry, O. H., Rosebrough, N. J., Farr, L. & Randall, R. J. *J. Biol. Chem.* 193, 265-275 (1951).

A virus-induced non-virion antigen specific for transformation at the surface of RSV-transformed fibroblasts

NEW antigenic specificities expressed at the surface of virus-transformed cells are considered to be due to the insertion of virus structural proteins into the plasma membrane (for review see ref. 1). However, there is evidence for the non-identity of some tumour antigens at the surface of virus-transformed cells with virion proteins²⁻⁵. For example, in the avian system, Rous sarcoma virus (RSV)-induced chicken sarcomas express at their surface a tumour-specific surface antigen (TSSA). The idea that the TSSA is not a virus structural protein, although under control of a viral gene, has been debated^{3,6-8}. We report here our studies on the antigenic composition of hamster fibroblasts

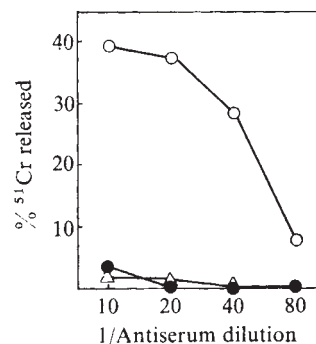


Fig. 1 Cytotoxic activity of rabbit anti-VCSA serum on control (●), RSV-transformed (○) or polyoma-transformed (△) BHK-C13/21 fibroblasts. The antiserum was raised in rabbits immunised with 2×10^7 live B4 cells in complete Freund's adjuvant and boosted twice at 30-d intervals. After pre-adsorption with an equal volume of packed sheep erythrocytes, the antiserum was chromatographed through a column of calf serum insolubilised by covalent binding to Sepharose 4B to remove contaminant anti-calf serum antibodies²⁶. Adsorption was carried out by incubating three times sequentially 2×10^7 C13 cells with 1 ml of antiserum for 30 min at 0 °C. The treatment was repeated with Py cells. The cytotoxicity assay¹⁰ was carried out by incubating 5×10^4 ^{51}Cr -labelled target cells for 60 min with 50 μl of the appropriate antiserum dilution and 50 μl of diluted guinea pig serum as the source of complement. The % of ^{51}Cr specifically released was calculated as follows:

$$\frac{\text{c.p.m. (experimental)} - \text{c.p.m. (blank)}}{\text{c.p.m. (maximum)} - \text{c.p.m. (blank)}} \times 100$$

Blanks were obtained by incubating cells with complement alone or with antiserum alone; the maximum amount of radioactivity released was measured by disrupting cells with Nonidet P-40 (5%).

transformed *in vitro* by RSV mutants, either defective for the synthesis of envelope glycoproteins (*env*⁻) or temperature-sensitive for the ability to transform the host cell (*ts*). We found that these cells expressed at their surface a tumour-specific antigen with the following properties: (1) it is expressed at the surface of hamster cells transformed by RSV but not by unrelated RNA or DNA oncogenic viruses; (2) it is expressed by RSV-transformed cells of different animal species; (3) it is not one of the known structural virion proteins; (4) in the temperature-sensitive mutant-transformed cells its expression correlates with the expression of the transforming gene.

We used a xenogenic antiserum raised in rabbits by immunisation with living BHK-C13/21 hamster fibroblasts transformed by the *env*⁻ Bryan strain of RSV (ref. 9) (B4). The antiserum was exhaustively adsorbed with the non-transformed parental hamster line (C13) and tested for complement-dependent cytotoxicity¹⁰ on target cells labelled with ^{51}Cr . After adsorption the antiserum had no cytotoxic activity against normal hamster cells, but still killed the transformed B4 cells. However, when tested in direct cytotoxicity or in adsorption experiments against hamster lines transformed by unrelated DNA or RNA tumour viruses (polyoma virus, SV40 and mouse sarcoma virus) and against Forssman positive sheep red blood cells¹¹, the antiserum was shown to contain antibodies directed against cross-reactive tumour-associated antigenic specificities and against Forssman antigen¹².

The antiserum was then further adsorbed with polyoma-transformed hamster fibroblasts and sheep red blood cells. After this second treatment, the antiserum reacted specifically against the B4 line used for immunisation (Fig. 1) and with other hamster lines transformed by RSV of a different subgroup (Table 1). Furthermore, positive results were obtained when it was tested against fibroblasts of different animal species transformed by RSV (rat and chicken) (Table 1). These experiments showed that the antigen recognised was common or cross-reactive to all RSV-

Table 1 Expression of VCSA at the surface of RSV-transformed fibroblasts

Targets	Species	Transformed by	Ref.	% ⁵¹ Cr released* ± sd
C13	Hamster	—	19	0
CEF†	Chicken	—	—	0
B4	Hamster	RSV (BH)	20	40 ± 5
RS 2/3	Hamster	RSV (SR-D)	21	42 ± 6
14-B	Hamster	RSV (SR-D)	16	41 ± 2
XC/t	Rat	RSV (PR-C)	22	30 ± 3
CEF-SR-A‡	Chicken	RSV (SR-A)	—	17 ± 6
CEF-PR-A‡	Chicken	RSV (PR-A)	—	31 ± 5
Py	Hamster	Polyoma virus	23	0
PM-1	Hamster	Mouse sarcoma virus	24	0
PM-3	Hamster	SV 40	25	0

*Complement-dependent cytotoxic activity of rabbit anti-VCSA serum tested at 1/10 dilution (maximum lysis) as described in Fig. 1 legend.

†CEF, secondary cultures of C/E chicken embryo fibroblasts.

‡C/E chicken embryo fibroblasts transformed *in vitro* by the Schmidt-Ruppin-A (CEF-SR-A) or the Prague-A (CEF-PR-A) strain of RSV.

transformed lines tested, indicating that its expression was under the control of the viral genome. For this reason we have called the doubly adsorbed antiserum anti-VCSA and the antigen identified VCSA (virus cell surface antigen).

To show that the VCSA is not a virus structural protein, we compared the cytotoxic activity on different RSV-transformed target cells of anti-VCSA serum with that of: an antiserum raised against detergent-disrupted purified virion particles of the RSV PR-C strain (anti-B77); a polyspecific antiserum raised against purified internal core proteins of the *gs* complex (anti-*gs*); a monospecific antiserum against purified gp85 (anti-gp85). No correlation was found between the cytotoxic activity of anti-VCSA serum and that of the three antisera specifically raised against virion structural proteins (Table 2). Moreover, as reported previously, specific binding of anti-VCSA radiolabelled immunoglobulins was observed with membrane antigens solubilised by the detergent NP-40 from B4 cells, whereas no binding was detectable with proteins solubilised by NP-40 from isolated RSV virions of subgroups A, B or C (refs 12,13).

Further experiments were carried out by testing the anti-VCSA serum on chicken embryo fibroblasts either transformed by RSV or infected by the transformation-defective Rous associated virus-1 (RAV-1). As chicken cells are permissive for virus replication, virus structural proteins were synthesised in both conditions and mature virions assembled and released from the cell surface. As shown in

Table 2 Expression of VCSA and virion structural proteins at the cell surface of RSV-transformed fibroblasts

Targets	anti- <i>gs</i>	% ⁵¹ Cr released (± sd) by anti-gp85	anti-B77	anti-VCSA
C13	0	0	0	0
B4	0	0	0	45 ± 1
RS 2/3	0	0	1 ± 0.5	43 ± 3
14-B	3 ± 1	5 ± 1	8 ± 3	40 ± 2
CEF	—	—	0	0
CEF-SR-A	—	—	31 ± 2	17 ± 6
CEF-PR-A	—	—	39 ± 2	27 ± 2
CEF-RAV-1	—	—	10 ± 2	0

Complement-dependent cytotoxic activity of rabbit antisera raised against RSV virion proteins and VCSA. The assay was carried out as described in the legend of Fig. 1 at antiserum dilution giving maximum lysis. The specificity of the antisera is described in the text. The targets were the cell lines listed above. CEF-RAV-1 were C/E chicken embryo fibroblasts (*gs*⁻, *chf*⁻) infected by the transformation-defective Rous associated virus-1; successful virus infection was demonstrated by *gs*-antigen expression in complement fixation tests and by high susceptibility to lysis with antisera raised against RSV virions of subgroup A (data not shown).

Table 2, the anti-B77 serum showed cytotoxic activity for both RAV-1-infected and RSV-transformed cells. In contrast, the anti-VCSA serum was significantly cytotoxic for the transformed cells only. Control chicken embryo fibroblasts (*gs*⁻, *chf*⁻) were not lysed by either antiserum. These results were confirmed by competition and adsorption experiments carried out with the anti-VCSA serum and RSV-transformed or RAV-1-infected chicken embryo fibroblasts (M.P., F. Tato and P.M.C., in preparation).

The nature of the gene(s) coding for VCSA is unknown. Our experiments show that the VCSA is not a virion structural protein. However, its presence in different RSV-transformed hamster lines and in RSV-transformed cells from different species suggests that its expression is under the control of the viral genome. The only RSV gene not coding for virion structural proteins is the *sarc* gene, the gene responsible for the induction and the maintenance of neoplastic transformation (for review see refs 14 and 15). To investigate whether the expression of VCSA is controlled by this gene, we carried out cytotoxicity and adsorption experiments on the 14-B hamster cell line¹⁶ transformed by the temperature-sensitive mutant *ts* FU-19. This is a mutant of RSV-SR-D that shows in permissive cells a loss

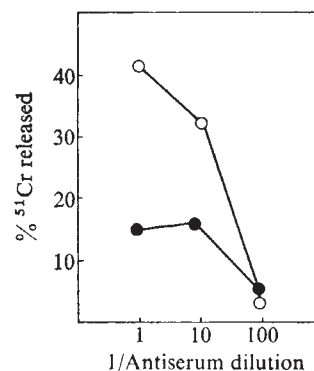


Fig. 2 Expression of VCSA at the surface of hamster fibroblasts transformed by a temperature-sensitive mutant of RSV. The cytotoxic activity of the anti-VCSA serum was tested on 14-B cells grown either at 37 °C (○) or at the restrictive temperature of 41 °C (●) for at least 12 h before the ⁵¹Cr-release assay. For more details see legend of Fig. 1.

of ability to transform the host cell at 41 °C (restrictive temperature), whereas it retains its capacity to replicate, showing that the only gene function sensitive to temperature is that of the *sarc* gene¹⁷. The 14-B cells, when grown at 41 °C, switch from the transformed to the normal phenotype¹⁸. The anti-VCSA serum lysed 14-B cells grown at 37 °C; however, when the temperature was raised to 41 °C for a minimum of 12 h, the degree of lysis was dramatically reduced (Fig. 2). These results were confirmed by testing the anti-VCSA serum against B4 cells after adsorption with 14-B cells grown either at 37 °C or at 41 °C. Those grown at 37 °C completely adsorbed the cytotoxic activity of the antiserum, while those grown at 41 °C were largely ineffective. By contrast, RS 2/3 cells, a clonal line of C13 fibroblasts transformed by the wild type of RSV-SR-D, adsorbed the cytotoxic antibodies when grown at either temperature (data not shown). These findings show the strict correlation between the appearance of VCSA at the cell surface and the expression of the *sarc* gene. The VCSA itself may be the product of the *sarc* gene; alternatively, it may be coded by a cellular gene under control of the *sarc* gene product. In the second hypothesis, the cellular gene involved would have been conserved during evolution to explain the immunological cross-reaction of VCSA observed in different animal species. Recently, a tumour-specific non-virion antigen of 60,000 molecular weight has been associated with the *sarc* gene expression in chicken and hamster transformed cells¹⁸. Its localisation within the cell is still unknown. The

elucidation of the relationship between this antigen and VCSA, which is expressed at the cell surface, will contribute to the understanding of the role played by the plasma membrane in malignant transformation.

This work was supported by research grants of the Italian National Research Council (C.N.R.) and the Ministry of Education. We thank M. Mura-Lagna and M. R. Amedeo for technical assistance. P.M.C. and M.P. hold a joint appointment with the University of Trieste School of Medicine. The anti-gs and anti-gp85 antisera were provided by Drs D. Bolognesi and M. Halpern.

PAOLO M. COMOGGIO
MARIA PRAT
MARILENA BERTINI

Department of Human Anatomy,
University of Torino Medical School,
10126 Torino, Italy

Received 29 December 1977; accepted 20 March 1978.

1. Kurth, R. *Nature* **256**, 613-615 (1975).
2. Law, L. W. & Appella, E. *Nature* **243**, 83-86 (1973).
3. Kurth, R. & Bauer, H. *Biochim. biophys. Acta* **417**, 1-23 (1975).
4. Essex, M. *Adv. Cancer Res.* **21**, 175-203 (1975).
5. Fenyo, E. M. & Klein, G. *Nature* **260**, 355-357 (1976).
6. Kurth, R. & Macpherson, I. A. *Nature* **264**, 261-263 (1976).
7. Rohrschneider, L. R., Bauer, H. & Bolognesi, D. P. *Virology* **67**, 234-241 (1975).
8. Aupoix, M. & Vigier, P. *Int. J. Cancer* **18**, 787-797 (1976).
9. De Giulio, C., Kawai, S., Dales, S. & Hanafusa, H. *Virology* **66**, 253-257 (1975).
10. Wright, P. W. & Law, L. W. *Proc. natn. Acad. Sci. U.S.A.* **68**, 973-977 (1971).
11. Vigier, P. & Bataillon, G. *Virology* **45**, 313-316 (1971).
12. Comoglio, P. M., Prat, M. & Bertini, M. in *Tumor-associated Antigens and their Specific Immune Response* (eds Arnon, R. & Spreafico, F.) (Academic, New York, in the press).
13. Prat, M. & Comoglio, P. M. *J. Immun. Meth.* **9**, 267-273 (1976).
14. Weiss, R. *Nature* **260**, 93 (1976).
15. Coffin, J. M. *Cancer Res.* **36**, 4282-4288 (1976).
16. Aupoix, M., Biquard, J. M. & Cachard, A. *Int. J. Cancer* **14**, 611-618 (1974).
17. Biquard, G. & Vigier, P. *Virology* **47**, 444-455 (1972).
18. Brugge, J. & Erikson, R. *Nature* **269**, 346-348 (1977).
19. Stoker, M. & Macpherson, I. A. *Nature* **203**, 1355-1357 (1964).
20. Pitts, J. in *Growth Control in Cell Culture* (eds Wolstenholme, G. & Knight, J.) 261-266 (Churchill, Edinburgh, 1971).
21. Montagnier, L. & Vigier, P. *C. r. hebdo. Seanc. Acad. Sci., Paris* **264**, 193-196 (1972).
22. Svoboda, J., Chyle, P., Simkovic, D. & Hiebert, L. *Folia biol. Praha* **9**, 77-81 (1963).
23. Stoker, M. *Virology* **18**, 649-658 (1962).
24. Monti-Bragadin, C. & Ulrich, K. *Int. J. Cancer* **9**, 383-392 (1972).
25. Monti-Bragadin, C., Conventi, C. & Meloni, G. *Boll. Soc. ital. Biol. sper.* **95**, 565-569 (1970).
26. Tarone, G. & Comoglio, P. M. *Expl Cell Res.* **110**, 143-152 (1977).

Immunoprevention of naturally occurring endogenous murine type-C RNA viruses

GENETIC sequences coding for type-C RNA viruses exist within the DNA of mouse cells^{1,2}. In some inbred mouse strains endogenous viruses infectious for mouse cells are spontaneously activated and replicate in the animal: there is an associated incidence of lymphoreticular neoplasia correlated with the age of onset and level of virus expression^{3,4}. Isolates from these naturally occurring lymphoreticular neoplasms as well as isolates activated from cells *in vitro* can directly induce lymphoreticular tumours in the same or an appropriately susceptible host strain^{3,5-8}. These findings have led to the question of whether, after experimental immunisation, the host might be able to control or prevent tumours associated with these endogenous viruses. Irradiation induced thymomas of C57BL/6 mice are of probable endogenous type-C virus aetiology^{9,10} and an antiviral immunity can be induced in these mice by active or passive means which results in a significant reduction in endogenous type-C virus expression and tumour incidence¹¹. Similar results have been obtained by Huebner and co-workers by induction of antiviral immunity in AKR mice^{12,13}. We have examined induction of immunity to endogenous type-C viruses in three inbred mouse strains, BALB/c, NIH Swiss and C57BL, known to differ in their immunological responsiveness¹⁴ as well as their expression of endogenous type-C virus. Inactivated murine ecotropic type-C virus was found to be effective in preventing the natural expression of endo-

genous ecotropic as well as the immunologically related xenotropic viruses. These findings have important implica-

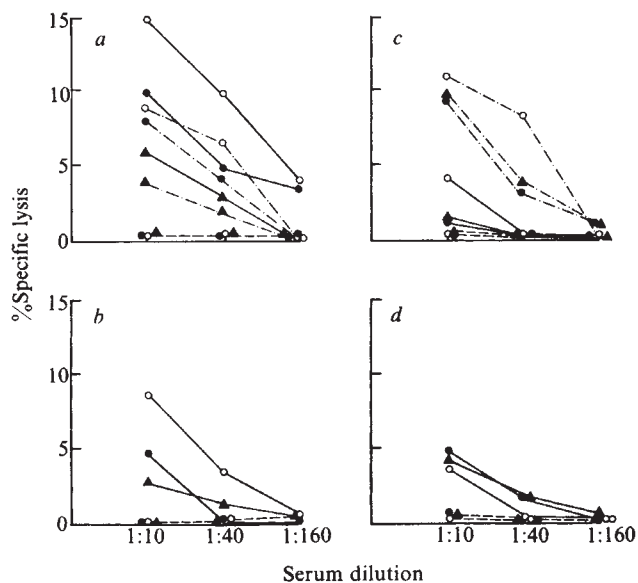


Fig. 1 Humoral cytotoxic immune responses of mice to inactivated Rauscher (a, b) and Gross virus (c, d) immunogens on the day of virus isolation. Serum titres shown are the means of three pools of sera per group; each pool made up of five to nine individual mouse sera. a And c, serum from C57BL/6 mice assayed against R-WM-7 (○—○), B-WM-7 (○—○), WM-7 (○—○) target cells. Serum from NIH Swiss mice assayed against R-WM-7 (●—●), B-WM-7 (●—●), WM-7 (●—●) target cells. Serum from BALB/c mice assayed against R-WM-7 (▲—▲), B-WM-7 (▲—▲), WM-7 (▲—▲) target cells. b And d, serum from C57BL/6 mice assayed against A673-class II (○—○), A673 (○—○) target cells. Serum from NIH Swiss mice assayed against A673-class II (●—●), A673 (●—●) target cells. Serum from BALB/c mice assayed against A673-class II: (▲—▲), A673 (▲—▲) target cells.

tions in the potential for prevention of naturally occurring neoplasms by antiviral immunity.

Endogenous type-C viruses of the mouse have been divided into three distinct classes based on immunological criteria¹⁵. Class I endogenous viruses are infectious for mouse cells, and are termed ecotropic¹⁶; it is these viruses especially in the BALB/c mouse that have been implicated as the causative agents of naturally occurring lymphoreticular neoplasms^{4,7}. The other two classes of endogenous murine type-C viruses are noninfectious for mouse cells and are termed xenotropic. These viruses have been found by infectivity or immunological assays in high frequency but at low levels in naturally occurring lymphoreticular and non-lymphoreticular tumours, as well as normal lymphoreticular tissues in the BALB/c and NIH Swiss mouse¹⁷.

Formalin-treated, Tween-80-ether-disrupted Rauscher leukaemia virus (R-MuLV) or Gross leukaemia virus (G-MuLV) immunogens were prepared as previously described^{11,18} and given to mice of each of the three inbred strains (subcutaneously on the first injection and intraperitoneally thereafter) on days 0, 14, and 28 at 200 µg, 100 µg, and 100 µg doses, respectively, followed by 50 µg doses administered at 2-4 week intervals (total of 12 immunisations). Four immunogen and control groups were included for each mouse strain as shown in Table 1. All mice were killed 3 weeks after the last immunisation, sera were collected for humoral cytotoxicity assays and the spleens were removed for virus isolation, cellular cytotoxicity assays and for viral antigen determinations.

The humoral and cellular immune cytotoxicity assays were performed by methods previously published^{19,20}. Target cells were a virus-free BALB/c syngeneic tumour cell line,

Table 1 Class I ecotropic virus and viral structural proteins in spleens of mice immunised with inactivated R-MuLV and G-MuLV

Mouse strain	Immunogen	No. of animals tested	1st Passage		3rd Passage		Class I MuLV p30 and p12 expression
			Class I MuLV titre	Class I MuLV incidence	Class I MuLV incidence	Class I MuLV incidence	
BALB/c	R-MuLV*	28	10 ^{1.1} †	2‡	3‡	2§	
	G-MuLV	26	10 ^{0.1}	1	2	0	
	CFA	14	10 ^{2.0}	10	11	10	
	Saline	15	10 ^{2.1}	12	12	11	
C576L/6	R-MuLV	26	< 10 ^{0.1}	0	1	0	
	G-MuLV	25	10 ^{0.3}	1	1	0	
	CFA	15	10 ^{1.6}	4	5	3	
	Saline	15	10 ^{1.3}	2	4	2	

*Sucrose banded and concentrated R-MuLV or G-MuLV were inactivated by formalin treatment and Tween-80-ether disruption^{11,18} and were given with complete Freund's adjuvant (CFA) on the first injection and with saline in subsequent injections.

†Lymphocyte suspensions from spleens were inoculated at 5×10^6 cells per plate on to 24 h cultures containing DEAE-dextran-treated SC-1 wild mouse embryo cells. Plaques were scored on two plates per inoculum by the XC assay procedure²⁵ at 7 d. Titres given are the mean titres of the virus positive animals in each group. The virus negative animals are disregarded for this computation.

‡No. of inocula yielding virus. Passages were made by scraping the cells from one tissue culture plate into 0.6 ml of culture supernatant and disrupting the cells. Fresh duplicate 24 h plates of SC-1 cells were then inoculated with 0.2 ml of the cell lysate and then tested 5 d later by the UV-XC assay.

§No. positive by competition radioimmunoassay²⁶. Spleen preparations containing < 10 ng p30 viral protein per mg of tissue, or < 5 ng p12 viral protein per mg of tissue were scored as negative. In each instance spleens scored as positive expressed levels of p30 of at least 80 ng per mg and levels of p12 of at least 40 ng per mg. Complete coordinate expression was observed for these two viral structural proteins in all animals tested.

WM-7 (ref. 21). Subcultures of this cell line were infected with R-MuLV and a naturally occurring B-tropic virus of the BALB/c mouse and designated R-WM-7 and B-WM-7, respectively. Other target cells included the non-infected human cell line A673 (ref. 13) and subcultures of A673 which were infected with the class II xenotropic virus²². Figure 1 shows that all three strains of mice responded with a virus-specific cytotoxic humoral immune response to both the R-MuLV and G-MuLV immunogens. Sera from the C57BL mouse were the most reactive, and sera from the BALB/c mouse least reactive. A predominant type-specific activity was seen with both the R-MuLV and the G-MuLV immunogens, however, a greater degree of cross-reactivity was seen in the former which included reactivity

to B-WM-7 as well as to cells shedding the class II xenotropic virus (A673-class II). Although yielding high activity to B-WM-7, the G-MuLV immunised animals were much less reactive to R-WM-7 and A673-class II. Non-infected control target cells were non-reactive in all the cytotoxicity assays. At the time of killing, lymphocytes from all three strains of immunised mice were non-reactive in cell-mediated cytotoxicity assays against the target cells described above.

To determine the effect of the viral immunisation on the incidence and titre of endogenous ecotropic and xenotropic type-C viruses, spleen lymphocyte suspensions were co-cultivated with SC-1 wild mouse embryo cells²³ and mink embryo cells²⁴, respectively. Ecotropic virus-induced

Table 2 Class II xenotropic virus and class III xenotropic viral structural proteins in spleens of mice immunised with inactivated R-MuLV and G-MuLV

Strain	Immunogen	No. of animals tested	Xenotropic class II virus incidence	Xenotropic class III virus p30 and p12 incidence	Xenotropic class III virus p30 and p12 levels			
					Mean	Range	Mean	Range
BALB/c	R-MuLV*	28	27†	28‡	18§	(10-40)	8§	(4-15)
	G-MuLV	26	25	26	20	(10-80)	10	(4-30)
	CFA	27	27	26	30	(10-160)	12	(4-60)
	Saline	29	27	29	30	(10-80)	12	(4-30)
C57BL/6	R-MuLV	26	6	26	6	(5-10)	3	(2-4)
	G-MuLV	29	11	28	12	(5-20)	6	(2-8)
	CFA	27	13	27	12	(5-30)	6	(2-10)
	Saline	26	15	26	14	(5-30)	6	(2-12)
NIH Swiss	R-MuLV	23	NT	23	35	(10-40)	15	(4-18)
	G-MuLV	24	NT	24	40	(10-80)	16	(4-30)
	CFA	24	NT	24	40	(10-80)	15	(4-40)
	Saline	21	NT	21	35	(20-40)	16	(6-20)

*Immunogen prepared and given as described in Table 1.

†No. of inocula yielding virus at passage three. Lymphocyte suspensions from spleens were inoculated at 5×10^6 cells per plate on to 24 h cultures containing DEAE-dextran-treated mink lung cells. Passages were made at 7 and 14 d by scraping the cells from one tissue culture plate into 0.6 ml of culture supernatant, disrupting the cells and inoculating fresh mink lung cells²⁴ or at the third passage S + L-mink cells were inoculated and plaques were scored at 7 d.

‡Spleens containing > 2 ng p30 per mg cell protein or > 2 ng p12 per mg cell protein were scored as positive. Complete coordinate expression was obtained for these two viral structural proteins in all animals tested.

§Of viral p30 or viral p12 (ng) per mg cell protein as determined by competition radioimmunoassay²⁶.

plaques were scored after 7 d and after two subsequent subcultures on to fresh SC-1 cells at 14 and 21 d by the XC assay²⁵. Xenotropic virus titres were determined by reverse transcriptase activity on the supernatant fluids at 7 d and after subculture at 14 d, and by the S+L-mink cell plaque assay at 21 d performed on cell lysates and supernatant fluids from the subcultured mink lung cells. Table 1 shows that both immunogens, inactivated R-MuLV or G-MuLV, were very effective in inducing an immunity to naturally occurring ecotropic virus in the BALB/c mouse. Infectious ecotropic virus was detected in only 5 of 54 immunised animals in the XC assays performed at the third passage as compared with 23 or 29 control animals. At the first passage only 3 of 54 animals yielded isolatable virus and the mean titres of these positives were very much less than the titres of isolates from the control group. To rule out the possibility that the inability to isolate virus from the immunised animals was caused by inhibitors in the inocula such as neutralising antibody, a direct measurement of the MuLV-p30 and the MuLV-p12 antigen content of the spleen cells was performed by competition immunoassay²⁶. Table 1 shows that p30 and p12 expression in the spleens was very closely correlated with ability to isolate virus. Like the BALB/c mouse the immunised C57BL mouse yielded very few ecotropic virus isolates at low titres; however, the incidence of isolatable virus from the control C57BL mice was much less than the BALB/c mice. Table 2 summarises the isolation incidence data at the third passage of the class II xenotropic virus and the class III xenotropic virus antigen incidence and titres. The immunised animals did not show a marked reduction of endogenous xenotropic virus activity as was found with the ecotropic virus activity. The R-MuLV-immunised C57BL mice, however, showed a trend of decreased activity in both the infectivity and antigen assays, which was statistically significant in the case of the class II virus incidence when compared to both control groups. The activity of the xenotropic viruses in the immunised BALB/c and NIH Swiss mice by both infectivity and antigen assays was not different from the control group (Table 2).

Hyperimmunisation of the mouse with inactivated type-C virus clearly induces an immunity to the endogenous ecotropic type-C virus(es). This immunity can be shown by immunoassays measuring both cytotoxic antibodies and precipitating antibodies¹⁴. More importantly, this regimen apparently induces an immunity that is effective in eliminating endogenous ecotropic virus and/or ecotropic virus-infected cells as they are expressed in the host. If this immunity can be sustained in the ageing animal we assume that it will be effective in preventing naturally occurring lymphoreticular neoplasms. Although correlated with humoral cytotoxic activity in this study, the effector immune mechanisms responsible for elimination of endogenous ecotropic virus are not as yet clearly defined. The low levels of endogenous complement in most mouse strains^{27,28} and the inability to demonstrate antibody mediated lysis *in vitro* of virus positive cells with mouse sera as a source of complement (M.K., unpublished) suggest that other immune mechanisms such as antibody-dependent cell-mediated immunity should be examined. Recent findings²⁹ have suggested that an aetiological relationship exists between spontaneous neoplasms in the AKR mouse and an endogenous type-C virus having genetically determined properties of xenotropic as well as ecotropic viruses. An etiological relationship of endogenous xenotropic viruses to naturally occurring tumours had not been previously shown, but its presence in tumours of all histological types had been noted¹⁷. The ability of the mouse to respond immunologically to its endogenous xenotropic viruses as reported here suggests that these newly isolated recombinant viruses^{29,30} should be examined to determine whether they

can immunologically affect the expression of endogenous type-C viruses and naturally occurring neoplasms in the mouse.

MEIR KENDE
JOHN R. STEPHENSON
GARY J. KELLOFF

Laboratory of RNA Tumor Viruses,
National Cancer Institute,
Frederick, Maryland 21701

ISMAIL K. AL-GHAZZOULI
MARSHALL DINOWITZ

Microbiological Associates,
Walkersville, Maryland 21793

Received 5 December 1977; accepted 20 March 1978.

1. Aaronson, S. A., Hartley, J. W. & Todaro, G. J. *Proc. natn. Acad. Sci. U.S.A.* **64**, 87-93 (1969).
2. Lieber, M. M. & Todaro, G. J. in *Cancer: A Comprehensive Treatise* **2** (ed. Becker, F. F.) 91-130 (Plenum, New York, 1975).
3. Peters, R. L., Spahn, G. J., Rabstein, L. S., Kelloff, G. J. & Huebner, R. J. *J. natn. Cancer Inst.* **51**, 621-630 (1973).
4. Lilly, F., Duran-Reynals, M. L. & Rowe, W. P. *J. exp. Med.* **141**, 882-889 (1975).
5. Gross, L. *Proc. Soc. exp. Biol. Med.* **78**, 342-343 (1951).
6. Lieberman, M. L. & Kaplan, H. S. *Science* **130**, 387-388 (1959).
7. Peters, R. L., Spahn, G. J., Rabstein, L. S., Kelloff, G. J. & Huebner, R. J. *Nature new Biol.* **244**, 103-105 (1973).
8. Stephenson, J. R., Greenberger, J. S. & Aaronson, S. A. *J. Virol.* **13**, 237-240 (1974).
9. Kaplan, H. S. *J. natn. Cancer Inst.* **9**, 55-56 (1948).
10. Kaplan, H. S. *Cancer Res.* **27**, 1325-1340 (1967).
11. Peters, R. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 1697-1701 (1977).
12. Huebner, R. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 620-624 (1976).
13. Huebner, R. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 4633-4635 (1976).
14. Stephenson, J. R. *et al. J. Virol.* **19**, 890-898 (1976).
15. Stephenson, J. R., Reynolds, R. K., Tronick, S. R. & Aaronson, S. A. *Virology* **67**, 404-414 (1975).
16. Levy, J. A. *Science* **182**, 1151-1153 (1973).
17. Kelloff, G. J., Peters, R. L., Donahoe, R. M., Stephenson, J. R. & Aaronson, S. A. *J. natn. Cancer Inst.* **57**, 85-89 (1976).
18. Kelloff, G. J. *et al. Cancer Res.* **36**, 622-630 (1976).
19. Herberman, R. B. & Oren, M. J. *J. natn. Cancer Inst.* **46**, 391-396 (1971).
20. Oren, M. E., Herberman, R. B. & Canty, T. G. *J. natn. Cancer Inst.* **46**, 621-628 (1971).
21. Al-Ghazzouli, I. K. *et al. J. Immun.* **117**, 2239-2248 (1976).
22. Aaronson, S. A. & Stephenson, J. R. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2055-2058 (1973).
23. Hartley, J. R. & Rowe, W. P. *Virology* **65**, 128-134 (1975).
24. Peebles, P. T. *Virology* **67**, 288-291 (1975).
25. Rowe, W. P., Pugh, W. E. & Hartley, J. W. *Virology* **42**, 1136-1139 (1970).
26. Oroszlán, S., Fisher, C. L., Stanley, T. B. & Gilden, R. V. *J. gen. Virol.* **8**, 1-10 (1970).
27. Borsos, T. & Cooper, M. *Proc. Soc. exp. Biol. Med.* **107**, 227-232 (1961).
28. Rosenberg, L. T. & Tachibana, D. K. *J. Immun.* **89**, 861-867 (1962).
29. Hartley, J. W., Wolford, N. K., Old, L. J. & Rowe, W. P. *Proc. natn. Acad. Sci. U.S.A.* **74**, 789-792 (1977).
30. Troxler, D. H., Lowy, D., Howk, R., Young, H. & Scolnick, E. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4671-4675 (1977).

T-T cell collaboration in rejection of a syngeneic SV40-induced sarcoma in mice

SYNERGISTIC or collaborative interactions between subpopulations of thymus-dependent lymphocytes were first demonstrated against alloantigens *in vivo* in graft-versus-host reactions¹, and subsequently *in vitro* in mixed lymphocyte culture responses² and in generation of cytotoxic lymphocytes³. At least two distinct subpopulations of T cells are involved in the interactions leading to collaborative responses, one acting as an amplifier or helper cell and the other type functioning as the precursor of the effector cell. The interactions between these two T-cell subpopulations yield effects greater than would be expected when each population is examined alone. It is now known that many tumours possess specific antigens which are recognised *in vivo* and *in vitro* by syngeneic hosts. There has been extensive study on immune response to SV40-induced syngeneic transplantable mKSA cells of BALB/c mice in our laboratory⁴⁻⁶. This tumour has been shown to possess tumour-associated antigens which can immunise specifically against the growth of mKSA cells. A very useful *in vivo* assay, the tumour cell neutralisation test (Winn assay), has been used successfully in our laboratory to measure the immune response to SV40 tumour associated antigens and to monitor the activity of various fractions

during biochemical antigen purification⁷⁻⁹. The rejection of the tumour was shown to be dependent on the presence of T cells⁸, however, it has not been established whether different subpopulations of T cells might be involved. There have been no reports on T-T cell collaboration *in vivo* or *in vitro* in immune responses to syngeneic tumours. The SV40 tumour system enabled us to investigate this important issue using the *in vivo* tumour cell neutralisation. Here we describe collaboration between subpopulations of T cells in the rejection of syngeneic SV40-induced tumour in mice.

Female BALB/c mice aged 8–12 weeks were inoculated subcutaneously (s.c.) with 1×10^6 mKSA-Tu5 tissue culture cells adopted from the ascites form of the tumour⁴. This tissue culture line has lost its oncogenic potential but has maintained tumour-specific transplantation antigen activity. Another group of mice was inoculated intraperitoneally with 5×10^6 mitomycin C-treated ($50 \mu\text{g/ml}^{-1}$ for 30 min at 37°C) Meth-A cells, a methylcholanthrene-induced tumour in BALB/c mice which has a strong tumour-specific transplantation antigen activity¹⁰. Pooled lymph nodes and thymus cells, 15–20 d after immunisation were incubated with 1×10^5 mKSA or Meth-A ascites cells for 45 min at 37°C and then injected s.c. into normal BALB/c mice. The animals were inspected until death from tumour growth. For depletion of thymus-derived cells, AKR anti-Thy 1.2 C3H antibody was provided by H. Holden (NCI). The preparation of this serum, the treatment of lymphoid cells and the tests for its specificity were performed using a technique detailed elsewhere¹¹. Representative experiments are shown in Tables 1–3; each experiment was repeated two or three times with similar results.

Table 1 Neutralisation of mKSA or Meth-A cells (Winn Assay) by effector cells

Lymphoid cells	Neutralisation of:	
	mKSA Dead/Total	Meth-A Dead/Total
Normal lymph node 200:1*	8/8	8/8
Normal thymus 200:1	8/8	8/8
Anti-mKSA lymph node 200:1	0/8	8/8
" 100:1	0/8	8/8
" 50:1	0/8	8/8
" 25:1	8/8	8/8
" 12:1	8/8	8/8
Anti-mKSA thymus 200:1	8/8	8/8
Anti-Meth-A lymph node 200:1	8/8	0/8
" 100:1	8/8	0/8
" 50:1	8/8	8/8
" 25:1	8/8	8/8
" 12:1	8/8	8/8
Anti-Meth-A thymus 200:1	8/8	8/8

* Effector: target cell ratio. The number of mKSA or Meth-A cells was kept constant, 1×10^5 .

In the first experiment various effector: target cell ratios of lymph node and thymus cells from normal BALB/c mice or mice immunised against mKSA or Meth-A cells plus mKSA or Meth-A cells were injected s.c. into normal BALB/c mice. All mice injected with normal lymph node or thymus cells died of mKSA or Meth-A tumour growth (Table 1). A dose-response relationship in neutralisation of mKSA cells was observed with graded numbers of lymph node cells immunised against mKSA. Complete protection against mKSA but not Meth-A cells was obtained with 50:1 or higher effector: target cell ratio. On the other hand, no prevention of mKSA tumour growth was detected with immune thymus cells at any effector: target cell ratio. Similarly no protection against the growth of Meth-A cells was observed with thymus cells immunised against Meth-A cells, but a dose-response relationship in neutralisation of Meth-A cells by increasing numbers of lymph node cells immunised against Meth-A cells was observed. In summary, no protection against mKSA cells was seen with 25:1 or

lower ratio of anti-mKSA lymph node: mKSA cells. No protection against Meth-A cells was seen with 50:1 or

Table 2 Collaborative effect of immune lymph node and thymus cells in rejection of mKSA cells

Lymph node cells	Thymus cells	Neutralisation of:	
		mKSA Dead/Total	Meth-A Dead/Total
Anti-mKSA 25:1*		8/8	8/8
	Anti-mKSA 200:1	8/8	8/8
Anti-mKSA 25:1	Anti-mKSA 200:1	0/8	8/8
" " 100:1		0/8	8/8
" " 50:1		0/8	8/8
" " 25:1		8/8	8/8
Anti-mKSA 12:1	Anti-mKSA 200:1	0/8	8/8
" " 100:1		0/8	8/8
" " 50:1		8/8	8/8
" " 25:1		8/8	8/8
Anti-mKSA 25:1	Normal 200:1	8/8	8/8
Anti-mKSA 25:1	Anti-Meth-A 200:1	8/8	8/8
Anti-Meth-A 50:1		8/8	8/8
	Anti-Meth-A 200:1	8/8	8/8
Anti-Meth-A 50:1	Anti-Meth-A 200:1	8/8	0/8
Anti-Meth-A 50:1	Anti-mKSA 200:1	8/8	8/8

* Effector: target cell ratio. The number of mKSA or Meth-A cells was kept constant, 1×10^5 .

lower ratio of anti-Meth-A lymph node: Meth-A cells. Thymus cells did not protect against either tumour at any cell number tested. In the second experiment the ability of immune lymph node and thymus cells to collaborate in rejection of mKSA cells was investigated. Anti-mKSA lymph node cells at suboptimal numbers were mixed with graded numbers of thymus cells and a constant number of mKSA or Meth-A cells and injected s.c. to normal BALB/c mice. Immune lymph node or thymus cells injected alone did not prevent tumour growth. However, after the lymphoid cells from both organs were mixed and injected together, complete prevention of tumour growth was observed (Table 2). This collaborative effect was specific. Similar results were obtained with anti-Meth-A lymphoid cells. In the last experiment the cell type needed for the collaborative effect was investigated. Untreated anti-mKSA immune lymph node cells at optimal numbers prevented mKSA tumour growth, as did cells treated with complement alone (Table 3). The activity of the cells was abrogated after their treatment with specific anti-Thy-1.2 serum and complement, which demonstrated that the cell type in the immune lymph nodes needed to neutralise mKSA is T cell-dependent. Untreated immune lymph node or thymus cells at suboptimal numbers did not neutralise mKSA when injected alone; however, they did prevent tumour growth when mixed and injected together, as shown by the previous experiments. This collaborative activity was abrogated

Table 3 Effect of Anti-Thy-1.2 serum and complement on the collaborative activity of lymph node and thymus cells

Lymph node Immune cells	Immune thymus cells	Neutralisation of:	
		mKSA Dead/Total	
Untreated 200:1*		0/8	
Anti-Thy-1.2 + C' 200:1		8/8	
C' 200:1		1/8	
Untreated 25:1		8/8	
	Untreated 200:1	7/8	
Untreated 25:1	Untreated 200:1	0/8	
Anti-Thy-1.2 + C' 25:1	Untreated 200:1	8/8	
C' 25:1	Untreated 200:1	0/8	
Untreated 25:1	Anti-Thy-1.2 + C' 200:1	7/8	
Untreated 25:1	C' 200:1	1/8	

* Effector: target cell ratio. The number of mKSA cells was kept at 1×10^5 .

after pretreatment of either lymph node or thymus cells with anti-Thy-1.2 serum and complement but not with complement alone before mixing and injecting the cells, which showed that T cells in both lymph nodes and thymus were needed for the collaborative activity.

The purpose of this study was to test the concept of T-T cell interactions during cell-mediated immune responses to tumour-associated antigens *in vivo*. The assay and the system used were the tumour cell neutralisation test (Winn assay) in the syngeneic SV40-induced sarcoma in BALB/c mice. The results show that there is cooperative cell interaction between thymocytes and peripheral T cells in cytotoxic responses *in vivo*. To our knowledge no similar phenomena have been previously reported in a syngeneic tumour system. These results are reminiscent of those reported by Asofsky *et al.* in studies of the cells that initiate graft-versus-host responses in F₁ neonatal mice¹. In an elegant hypothesis these authors suggested that different maturation stages of T lymphocytes exist (termed T₁ and T₂) and that cell collaboration between T₁ (precursor cells of effector cells) and T₂ (amplifier cells) takes place. The observed phenomenon closely resembles also the synergism between T-cell subpopulations in *in vitro* lymphocyte proliferative response² and generation of cytotoxic reactivity³ to alloantigens. The interactions between these two T-cell subpopulations yield effects greater than would be expected when each subpopulation is examined alone. In the present study, it was only possible to observe whether collaborative activity existed (rather than synergistic), as it is difficult to quantitate immune response by *in vivo* measurement. It was clear, however, that while suboptimal numbers of either T-cell population alone did not prevent tumour growth, combinations of both did. The mechanism of T-T cell interaction during allograft responses or in responses to syngeneic tumours is unknown. It was suggested in *in vitro* allograft system that T₁ cells (thymocytes, short lived T cells) respond mainly against allogeneic LD differences, T₂ cells (long-lived recirculating T cells) react immunologically specific against SD (H-2) antigens and differentiate into cytotoxic effector cells. The proliferative response of T₁ cells to LD differences results in an amplification of a T₂ anti-SD (H-2) reaction. The mechanism of amplification may be mediated by a soluble mediator produced by T₁ cells¹². It remains to be determined whether a similar mechanism also operates in syngeneic tumour systems and if there is any relevance of T-T cell collaboration in relation to tumour growth and regression.

MOSHE GLASER*

LLOYD W. LAW

Laboratory of Cell Biology,
National Cancer Institute,
Bethesda, Maryland 20014

Received 5 January; accepted 13 March 1978.

*To whom reprint requests should be addressed.

1. Asofsky, R., Cantor, H. & Tigelaar, R. E. *Prog. Immun.* **1**, 369-381 (1971).
2. Tiltan, W., Gerbare-Delima, M. & Walford, R. L. *J. exp. Med.* **139**, 1488 (1974).
3. Cohen, L. & Howe, M. L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2707-2710 (1973).
4. Drapkin, M. S., Appella, E. & Law, L. W. *J. natn. Cancer Inst.* **52**, 259-264 (1974).
5. Henriksen, O., Law, L. W. & Appella, E. *J. natn. Cancer Inst.* **58**, 1785-1788 (1977).
6. Dean, J. H., McCoy, J. L., Lewis, D., Appella, E. & Law, L. W. *Int. J. Cancer* **16**, 465-475 (1975).
7. Howell, S. B., Esber, E. C. & Law, L. W. *J. natn. Cancer Inst.* **52**, 1361-1363 (1974).
8. Howell, S. B., Dean, J. H., Esber, E. C. & Law, L. W. *Int. J. Cancer* **14**, 662-674 (1974).
9. Law, L. W., Henriksen, O. & Appella, E. *Nature* **257**, 234-235 (1975).
10. Natori, T., Law, L. W. & Appella, E. *J. natn. Cancer Inst.* **59**, 1331-1333 (1977).
11. Herberman, R. B., Nunn, M. E., Lavrin, D. H. & Asofsky, R. *J. natn. Cancer Inst.* **5**, 1509-1512 (1973).
12. Plate, J. M. *Nature* **260**, 329-331 (1976).

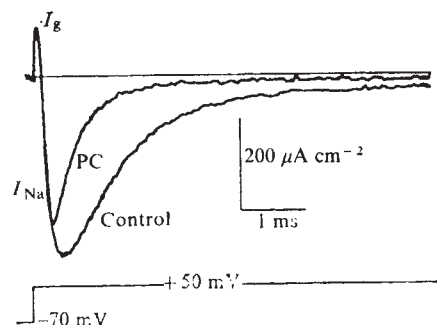
Immobilisation of gating charge by a substance that simulates inactivation

THE ionic pores of nerve membranes have voltage-sensitive gates which control not only ion movements through the pores, but also the access of various drugs to receptors or blocking sites within the pores. One blocking agent with a gate-protected receptor is the quaternary nitrogen compound pancuronium. As shown by Yeh and Narahashi¹, it can enter and block sodium pores when applied internally, but it can only equilibrate with its receptor if the pore is activated. Equilibration requires an appreciable fraction of a millisecond, so that pores allow passage transiently before they are blocked. Once in an open pore, a pancuronium ion has a 'foot in the door' action and prevents closing of both the activation and inactivation gate of the pore for as long as it remains in its blocking site. Yeh and Narahashi made their observations by studying ionic current through sodium pores. It is also possible to obtain information about sodium channel gates by measuring gating current, which is a small current generated by movement within the membrane of the charged structures that open and close the pores². We carried out the studies reported here to ascertain whether pancuronium had the effect on gating current that would be anticipated from its action on I_{Na} , and we report here that it does. 'On' gating current, which occurs as the channels are opening and before pancuronium enters, is unaffected, whereas 'off' gating current is slowed by the 'foot in the door' effect, and consequently reduced in amplitude. Our results provide additional evidence that gating current is in fact associated with gating of the sodium pores. Also, they provide a good clue to the nature of that component of the gating current which does not inactivate.

The experiments were carried out on voltage-clamped, internally perfused giant axons of the squid *Loligo pealei*. The internal solution contained 200 mM Cs⁺, 100 mM glutamate, 100 mM fluoride, 10 mM Tris and 560 mM sucrose (pH 7.2). The external solution contained either 150 mM NaCl, 320 mM Tris-HCl and 50 mM CaCl₂ (pH 7.0) for $I_{Na}+I_{K}$ (sodium and gating current) measurements; or 480 mM Tris-HCl, 50 mM CaCl₂ and 10⁻⁷ M tetrodotoxin (pH 7.0) for gating current measures. The linear portion of capacitative current was eliminated by subtracting the current from control pulses applied in a voltage range in which no ionic channels were opened or closed (P/4 procedure; see ref. 2 for details). Pancuronium was added to the internal solution at a concentration of 1 mM.

Figure 1 shows the normal pattern of $I_{Na}+I_{K}$ recorded during a pulse from -70 to +50 mV, and the effect of

Fig. 1 Pancuronium does not affect the 'on' gating current (I_g) or the rising phase of the sodium current (I_{Na}). The rapid decay of the I_{Na} in the trace labelled PC results from the entry of pancuronium (PC) into activated channels. Control, no pancuronium. Each trace is the average current for 10 pulses from -70 to +50 mV, minus the average current of 40 control pulses from -150 to -120 mV (not shown).



pancuronium on this pattern. The initial upward deflection is the gating current that is associated with the opening of the sodium pores. This is followed by an inward sodium current that increases in magnitude for approximately 800 μ s as the activation gates of sodium pores open, and then decays as the inactivation gates of the channels close. The addition of pancuronium accelerated the decay of I_{Na} and the trace gives the impression that the closing of the inactivation gate has been speeded up. It has been shown, however, that the decay of current in the presence of pancuronium reflects the entry of drug molecules through the open pores, and that the inactivation gate of a pancuronium occluded channel does not close (ref. 1, and see below). Both 'on' gating current and the rising phase of I_{Na} are unaffected by pancuronium, which is consistent with the hypothesis that closed pores do not contain the drug, and that it enters only after the pores have opened.

Analysis of the action of pancuronium is complicated by the presence of inactivation, and to avoid this complication we perfused our preparation internally with the enzyme mixture Pronase, which destroys inactivation but leaves the activation gate of surviving channels unaltered³. Figure 2a shows I_{Na} tail currents from a Pronase-treated axon as the sodium pores closed, in the presence and absence of internally applied pancuronium. The pores were first opened by a pulse from -70 to $+80$ mV for 0.3, 0.7 or 6 ms, and the tail currents were recorded as the pores closed on return to -70 mV. In the absence of pancuronium the tails were large and decayed rapidly. The 6-ms tail was somewhat smaller than the one at 0.3 ms because Pronase treatment was stopped when about 30 or 40% of inactivation remained intact. (Axons that were treated more severely with Pronase did not survive well.) In the presence of pancuronium the initial amplitude of the tail after 0.3 ms was reduced because pancuronium has blocked about two-thirds of the pores, and the subsequent decay of the current is slowed by the 'foot in the door' effect: after the 'foot' has been removed, a pore conducts for a time before closing. After 0.7 or 6 ms at $+80$ mV, most or all of the pores were blocked by pancuronium, and the current increased in magnitude for approximately 200 μ s from an initial amplitude of near zero, as pancuronium ions left the pores. The I_{Na} peaked and then decayed as the activation gates of the freshly cleared pores closed, but decay of current was slower than normal because the number of open pores was replenished by pores that were losing their blocking ions.

Fig. 2 The decay of the I_{Na} is slowed by pancuronium and the fast component of the 'off' gating current is reduced or abolished. The fibre was treated with Pronase, and about 60% of inactivation was destroyed. Current was averaged at the end of five pulses from -70 to $+80$ mV, with control pulses from -150 to -112.5 mV ($-70 \rightarrow +80 \rightarrow -70$; $-150 \rightarrow -112.5 \rightarrow -150$ mV). Pulse duration for each trace is indicated. $I_{Na} + I_g$ was first recorded, then I_g was measured (after removing sodium and adding tetrodotoxin) and subtracted to yield I_{Na} .

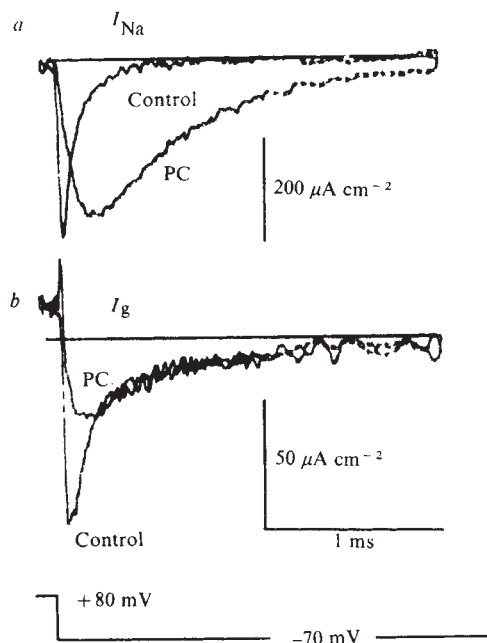
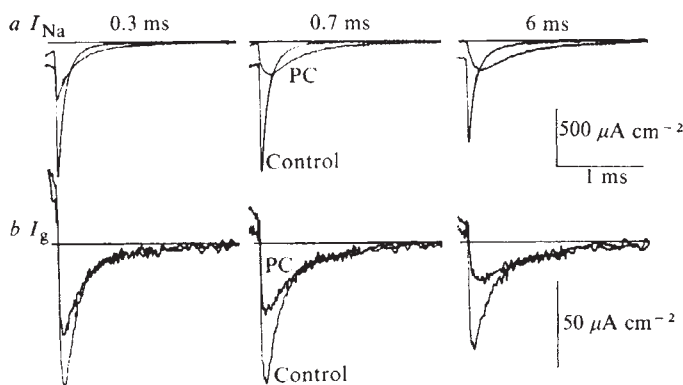


Fig. 3 In a fibre with intact inactivation, pancuronium in the pores prevents closing of the activation and inactivation gates (a) and abolishes the fast 'off' component of the I_g (b). The tails were recorded at the end of a 6-ms pulse from -70 to $+80$ mV (see Fig. 2 for details).

Gating currents were recorded using the same procedure after removing sodium ion from the medium and adding tetrodotoxin (Fig. 2b). The currents were inward, and reflected the return of gating charge to its resting or 'off' position. They had a fast component that was suppressed by pancuronium more or less in proportion to the drug's effect on I_{Na} (Fig. 2a); after a 6-ms pulse the suppression was more marked than after 0.3 ms. Our interpretation is that pancuronium blocks not only the closing of the activation gate, but also the charge movement that is associated with closing. Charge movement and closing seem to be tightly linked.

Axons with inactivation intact gave similar results. Figure 3a shows the I_{Na} with and without pancuronium as the pores closed after repolarisation from a 6-ms pulse to $+80$ mV. The I_{Na} was small in the control record (relative to Fig. 2a) because inactivation had blocked 75% of the sodium pores. With pancuronium present the current increased in magnitude from near zero as pancuronium ions came out of the pores, and then decayed slowly. The persistence and fairly large amplitude of the I_{Na} show that pancuronium in a pore prevents closing of both the activation and inactivation gate¹. The control gating current record in Fig. 3b has a fast component that is resistant to inactivation (about one-fourth of the gating charge would not be inactivated even if the inactivation of the I_{Na} were complete; see ref. 4). This component is eliminated by pancuronium, a fact that provides strong evidence that this component is the gating current of the sodium pores. Our results show clearly that pancuronium slows the 'off' gating current, and does not affect the 'on' current. In this respect they are in perfect agreement with studies of the effect of pancuronium on I_{Na} (ref. 1).

Pancuronium in many ways simulates inactivation, and it is reasonable to speculate that a pancuronium ion competes with the inactivating particle for a blocking site that must be approached from the inner end of the pore. Consistent with this is the fact that blockade and inactivation by pancuronium are mutually exclusive states (ref. 1; and Fig. 3a). There is, however, an important difference in that pores conduct during recovery from blockade by pan-

cunonium (Figs 2a and 3a) but not during recovery from inactivation'. Correlated with this is the difference in their effect on the 'off' gating current: pancuronium eliminates all of the fast component of the 'off' current, whereas inactivation affects only 65–75% of it (Fig. 3b). This suggests that the inactivation-resistant part of the 'off' current is associated with a transition that closes the pore so that it cannot conduct during recovery from inactivation'. Perhaps a pancuronium ion goes further into the pore than the inactivating particle and interferes more thoroughly with the activation-gating machinery.

There is one aspect of the data that requires further experimental examination. In Fig. 2b (in the presence of tetrodotoxin), if pancuronium came out of the pores as rapidly as it does in Fig. 2a (no tetrodotoxin), the time course of the I_{Ca} should be somewhat prolonged, but total charge movement should be the same with or without pancuronium. Instead, it is smaller. The ratios of the 'off' charge movement to 'on' in the absence of pancuronium (Fig. 2b) are 1.2, 1.0 and 0.83 respectively for the 0.3-, 0.7- and 6-ms duration pulses (for a baseline fitted between 1.7 and 2.7 ms after the 'on' or 'off' step). In the presence of pancuronium the ratios were 0.92, 0.82 and 0.5, distinctly smaller than expected, even if one allows a substantial margin for baseline uncertainty, as one must in a Pronase-treated axon. This may mean that pancuronium comes out of the pores more slowly when tetrodotoxin is blocking their outer ends, and that the slow movement of gating charge generates a current that is lost in the noise of the baseline.

This work was supported by NIH grants, NS 12547 and 14144.

J. Z. YEH

Department of Pharmacology,
Northwestern University,
Chicago, Illinois 60611

C. M. ARMSTRONG

Department of Physiology,
University of Pennsylvania,
Philadelphia, Pennsylvania 19104

Received 19 December 1977; accepted 20 March 1978.

1. Yeh, J. Z. & Narahashi, T. J. *gen. Physiol.* **69**, 293–323 (1977).
2. Armstrong, C. M. & Bezanilla, F. J. *gen. Physiol.* **63**, 533–552 (1974).
3. Armstrong, C. M., Bezanilla, F. & Rojas, E. J. *gen. Physiol.* **62**, 375–391 (1973).
4. Armstrong, C. M. & Bezanilla, F. J. *gen. Physiol.* **70**, 567–590 (1977).

Is digitalis inotropy associated with enhanced slow inward calcium current?

THE positive inotropic effect of digitalis compounds is generally attributed to an increase in the amount of activator calcium¹ and not to a change in the calcium sensitivity of regulatory contractile proteins^{2,3}. The main question is the cellular or subcellular basis of the increase in the myoplasmic calcium transient. Since calcium entry through the slow inward current may play an important part in excitation-contraction coupling, it has been suggested that the slow inward current might be enhanced by digitalis^{4,5}. All the evidence has been negative^{6–11} apart from a report by Dramane *et al.*¹² and results of H. Brown, W. Giles and S. Noble (personal communication). We have re-examined this question and have found that strophanthidin can increase the slow inward current and alter its re-priming kinetics in certain conditions. These observations may provide a partial explanation of the positive inotropic effect observed in the same experiments.

We studied the effects of strophanthidin on the electrical and mechanical activity of cardiac Purkinje fibres from calf hearts. The aglycone strophanthidin was preferred to cardiac glycosides such as ouabain because its effects are more readily reversible. Purkinje fibres were used because

recent work has characterised the influence of a wide range of digitalis concentrations on contractility¹³ and transport of potassium¹⁴ and sodium¹⁵ in this preparation. Total contractile force was measured in preparations less than 2 mm long using an Endevco 87102 transducer. Membrane current was measured with a conventional two-microelectrode voltage clamp technique¹⁶. In many experiments, the slow inward current was evoked by a step depolarisation from a holding potential near -40 mV to a test potential near -20 mV. These conditions favoured the appearance of slow inward current immediately following the depolarising step with a minimum interference from the fast sodium current¹⁷ and the transient outward current^{18,19}. A programmable digital stimulator provided command pulses for the voltage clamp and external shocks for evoked action potentials. The stimulator cycle was repeated every 30 s and consisted of a train of about 15 stimuli at 0.5–1.0 Hz followed by a steady clamp holding potential and depolarising pulse. This procedure was used to compare changes in action potential configuration, twitch force and membrane current following drug administration.

Figure 1 shows superimposed records of mechanical and electrical activity in the absence and presence of $1 \mu\text{M}$ strophanthidin. The records were obtained after a 3 min exposure, when the positive inotropic effect had clearly developed, but before the development of obvious toxicity. Thus, Fig. 1b shows a twofold increase in twitch amplitude over control without any aftercontractions^{20,21} following the twitch. Figure 1c and d show corresponding results under voltage clamp. Drug exposure altered the net membrane current associated with the fixed voltage waveform, a 2 s depolarisation from -38 mV to -21 mV. The late steady current at both potential levels was displayed in the inward direction, as found previously for the dihydroouabain²² and ouabain¹⁴. More significant in this case is the change in the time-dependent current, which reflects the activation and inactivation of the slow inward current²³. The downward shift of the early peak is much larger than the shift in late

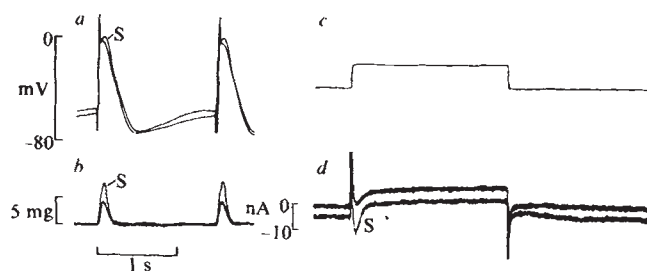


Fig. 1 Records of membrane potential, force and membrane current in the absence and presence of strophanthidin ($1 \mu\text{M}$, 3 min exposure). Action potentials (a) and twitches (b) were elicited by external stimuli. Shock artefacts appear as downward deflections preceding action potential upstrokes. Traces in (a) and (b) show the last two responses in a train of 14 beats evoked during the application of a steady hyperpolarising current of -15 nA. Strophanthidin (S) elevated the plateau and enhanced the diastolic depolarisation. The voltage clamp was imposed at the point where trace (a) ends and held the membrane potential at -38 mV for 2 s before the depolarising step shown in (c). The depolarising clamp pulse to -21 mV was associated with the current records in (d). Records were taken with a Brush 440 chart recorder and superimposed photographically. The action potential overshoots were attenuated by the recorder. The current signal was smoothed by a low-pass filter with an exponential time constant of 5 ms. Force traces which accompanied the voltage clamp pulse were omitted for clarity. The step depolarisation produced a twitch amplitude of 0.06 mg in the absence of drug, and 0.11 mg after exposure to strophanthidin for 3 min. (Preparation R13-1). The composition of the modified Tyrode solution used in this and other experiments was as follows (in mM): 150 Na, 4 K, 155.8 Cl, 5.4 Ca, 0.5 Mg, 10 Tris-maleate (pH 7.2–7.4), 10 glucose. The temperature was ~ 36 – 37°C and was held within $\pm 0.2^\circ\text{C}$ during a given experiment.

current. Thus it seems safe to conclude that the slow inward current is increased. The net membrane current record showed good recovery to the control after removal of the drug (data not shown). Another indication of the increase in slow inward current is given in Fig. 1a which shows a prominent enhancement of the secondary depolarisation during the plateau. Enhancement of peak slow inward current by strophanthidin was found in 13 of 14 preparations. These results suggest some resemblance between effects of cardiotonic steroids and sympathetic amines.

Enhancement of slow inward current by adrenergic compounds^{23,24} plays a major part in their positive inotropic

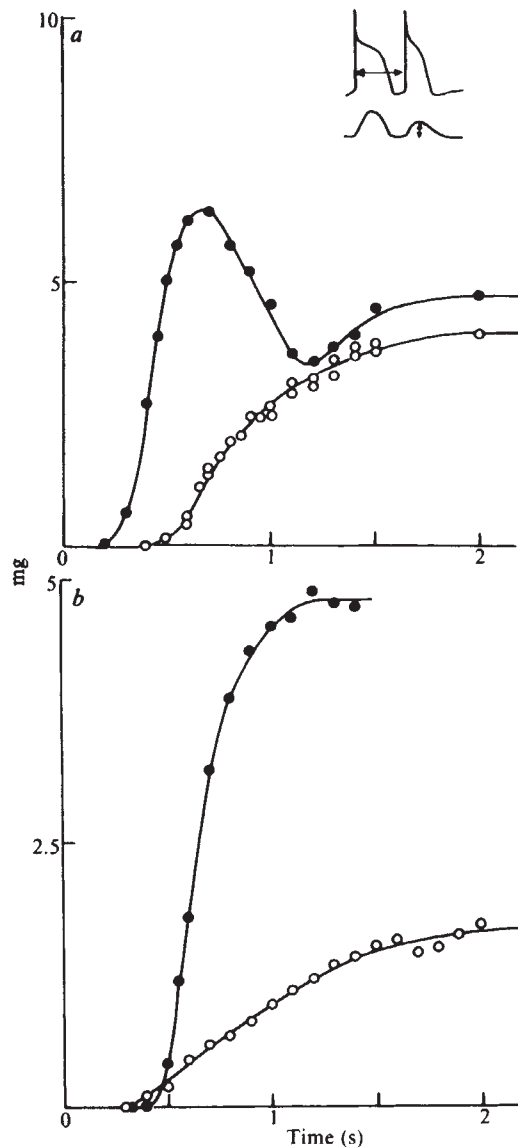


Fig. 2 Influence of strophanthidin and adrenaline on the restitution of contractility. Each point represents a single test contraction after a regular train of about 8 beats. Twitch amplitude (ordinate) is plotted against the interval between the last regular stimulus and the test stimulus. *a*, Control run (open symbols) and run beginning 14 min after exposure to 1 μ M strophanthidin (solid symbols). Train stimulus rate was 0.5 Hz in both runs. During the control run, maximum diastolic potential (MDP) was -93 mV with zero applied current. During strophanthidin runs, MDP was kept at -89 mV with -16 nA holding current. *b*, Control run taken after removal of strophanthidin (open symbols) and run beginning 3 min after exposure to 1 μ M adrenaline bitartrate (solid symbols). Note change in vertical scale relative to (*a*). Train stimulus rate was set at 1.0 Hz in both runs to avoid spontaneous impulses due to adrenaline. In both runs, MDP was -87 mV with zero applied current. (Preparation R30-1, apparent cylindrical surface area, 0.009 cm².)

effect²⁵. Figure 2 shows an important contrast between strophanthidin and adrenaline when their inotropic effects are studied in the same preparation. Recovery of contractile strength is plotted against the interval between beats. Trains of shocks were applied at constant frequency to achieve a steady twitch response. They were followed after a variable interval by an additional test stimulus. In Fig. 2 the amplitude of the test contraction is plotted against the interval. Figure 2*b* shows contractile restitution in the presence of 1 μ M adrenaline and restitution in a prior control run. Twitch force is increased by adrenaline at all intervals but the time course of restitution remains monotonic. This response differs from the effect of strophanthidin. The positive inotropic effect of the cardiotonic steroid depends on the duration of the immediately preceding inter-beat interval in a non-monotonic fashion (Fig. 2*a*). Restitution of contraction reaches a peak at 0.7 s, falls to a minimum at 1.2 s, then climbs to a steady level at longer intervals. Similar oscillations in contractile restitution were evident in other experiments and are in accord with previous observations in Purkinje fibres²⁶ and in ventricular muscle (see ref. 21 for review).

Can the oscillation in contractile recovery be related to changes in the slow inward current? Voltage clamp experiments in strophanthidin-treated preparations were carried out to examine this point (Fig. 3). Two identical depolarising clamp pulses were separated by a variable interval (Fig. 3*a*). The associated set of force records (Fig. 3*c*) shows the oscillatory dependence of contraction amplitude on interval. This oscillation is mirrored by the envelope of slow inward current peaks in panel B. The variations in slow inward current and twitch force show the same oscillatory period; the largest slow inward current and the strongest twitch are both elicited by the pulse following a 500 ms interval. This kinetic similarity reinforces the correlation between the positive inotropic effect and enhanced slow inward current. Oscillatory repriming of the slow inward current has not been reported previously for Purkinje fibres, but has been observed by Hiraoka and Sano³⁰ in dog ventricular muscle in the absence of digitalis.

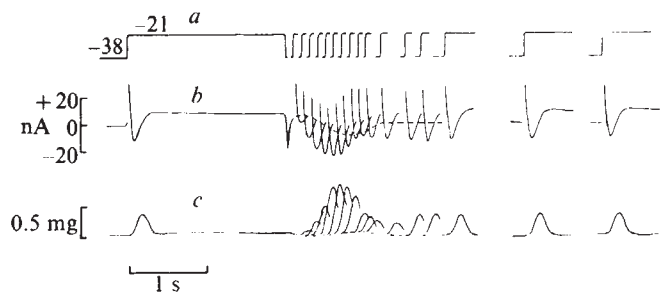


Fig. 3 Oscillatory repriming of slow inward current and twitch contraction in the presence of strophanthidin. A 2-s depolarising clamp pulse was followed after a variable interval by a second depolarising step (*a*). Lower panels show superimposed tracings of membrane current (*b*) and contractile force (*c*). The dashed traces are associated with repolarisation to -38 mV after the clamp pulse. Strophanthidin-induced transient inward current (TI)²⁷ appears in (*b*), with a small aftercontraction²¹ in (*c*). These oscillatory signals lag behind the oscillations in the magnitudes of the slow inward current and twitch force. The TI pathway can be distinguished from slow inward current channels²⁸ by its reversal potential (near -5 mV) and by the relative insensitivity of the reversal potential to changes in extracellular calcium²⁹. Variations in current carried by the TI at -21 mV should not interfere seriously with the envelope of slow inward current peaks: the TI driving force at -21 mV is roughly half that at -38 mV; the TI conductance is well short of its peak at the time of maximum apparent slow inward current. The phase lag between twitch restitution and aftercontraction in (*c*) is consistent with previous results in ventricular muscle²¹. Recordings were made in a run beginning 14 min after administration of 1 μ M strophanthidin. (Preparation R13-2.)

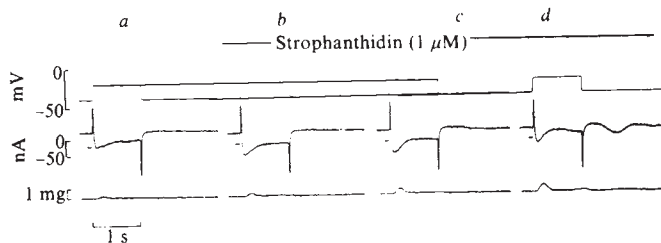


Fig. 4 Temporal separation between development of therapeutic and toxic effects at various times during continuous exposure to $1 \mu\text{M}$ strophanthidin. *a*, Control; *b-d*, 4, 8, 14 min after drug administration. Upper records show depolarising clamp pulses from a protocol similar to that used in Fig. 1. Associated current and force records are shown below. Horizontal bars indicate level of peak slow inward current in control. (Preparation R20-1.)

Most of our experiments relied on relatively high concentrations of strophanthidin to produce large, clear-cut effects. Prolonged exposure to such doses led eventually to arrhythmogenic actions³¹. It is important, therefore, to demonstrate that therapeutic effects on the magnitude and repriming of contraction can be set apart from toxic phenomena such as transient inward current²⁷ and after-contraction²¹. Figure 4 provides evidence for such a separation. The development of positive inotropic and toxic changes are compared during continuous exposure to cardiotonic steroid. Figure 4*a* shows the slow inward current and associated twitch in the absence of drug, and Fig. 4*b-d* show records taken at various times after administering $1 \mu\text{M}$ strophanthidin. The horizontal markings represent the level of the inward current peak in control. Peak slow inward current and twitch height are both increased after 4 min exposure. There is a downward displacement of late current during the pulse, as if residual slow inward current^{23,18,32} were increased. After 8 min, the peak current level remained unchanged, while the twitch continued to grow. After 14 min, the contraction increased even further, but the inward current peak fell off from its previous increase. Figure 4*d* also shows toxic manifestations: a small transient inward current and an aftercontraction following the termination of the depolarising pulse. The main point here is that neither of these indications of toxicity were seen following the clamp pulse in Fig. 4*b*, when the slow inward current was already maximally enhanced. The lack of parallelism between changes in slow inward current and twitch force in Fig. 4*c* and *d* is also interesting. It suggests that while changes in calcium current may contribute to the early inotropic effect, additional mechanism(s) must dominate at a later stage.

Further evidence for an operational separation between therapeutic and toxic actions was provided by low drug dose experiments. Figure 5 illustrates the influence of 20 nM strophanthidin on contractile restitution. The traces marked with vertical bars show the last two beats from trains of contractions at 1 Hz. The other superimposed traces were obtained in separate trials where the last inter-stimulus interval was varied. The envelope of superimposed contractions showed a smooth monotonic recovery in the control run (Fig. 5*a*) and became markedly oscillatory after maintained exposure to the cardiotonic steroid (Fig. 5*b*). The time course of contractile repriming showed good recovery when the drug was removed (data not shown). The development of oscillatory restitution was a consistent finding in other experiments using near-threshold concentrations of strophanthidin (20–50 nM), while aftercontractions and other toxic effects were not apparent. This dissociation does not rule out the possibility that oscillatory restitution and aftercontractions share similar underlying mechanisms²¹. Changes in contractile restitution might be especially important at low drug concentrations. For example, in Fig.

5*b*, the twitch was increased more than twofold following intervals between 0.5 and 1.8 s, but only about 20% after intervals between 5 and 8 s. The effects of low strophanthidin concentrations on contractile repriming suggest that slow inward current may also be enhanced. We examined the effect of 50 nM strophanthidin in an experiment using the protocol shown in Fig. 1, and found small increases in slow inward current, plateau height and twitch amplitude. These effects were qualitatively similar to those shown in Fig. 1. Additional experiments using low concentrations of cardiotonic steroid are underway.

The results presented here are consistent with previous tracer flux studies in which digitalis compounds promoted calcium uptake by working myocardium^{33,34}. They raise the possibility that some part of this extra calcium uptake may be electrically detectable. However, our experiments do not reveal the mechanism by which strophanthidin influences the slow inward current. It will be interesting to find out whether the enhancement of this current is related in some way to inhibition, stimulation, or some other effect³⁵ of cardiotonic steroids on the sodium pump mechanism.

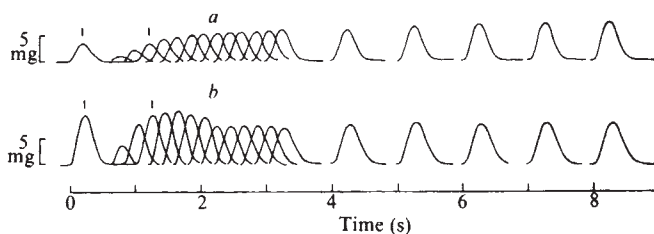


Fig. 5 Influence of a low, subtoxic concentration of strophanthidin on contractile repriming. *a*, Control run; *b*, between 9 and 20 min after administering 20 nM strophanthidin. Protocol similar to Fig. 2. Vertical bars mark steady-state contractions at the basic driving frequency of 1 Hz. Other traces are superimposed records of premature or postmature responses obtained in separate trials. Note that the positive inotropic effect is much more pronounced at intervals of 1–2 s than at longer intervals. (Preparation E2-1, experiment carried out with collaboration of E. Marban.)

Our results differ from earlier work in myocardial preparations which did not show enhancement by digitalis compounds of slow inward current^{9,10} or depolarising responses which depend on this current^{6–8,11}. Explanations such as differences in drug, species or tissue type cannot be excluded, but they seem unlikely since ouabain-induced increases in slow inward current have been found in frog atrial trabeculae by Dramane *et al.*¹² and by Brown, Giles and Noble (personal communication). A more likely cause of the conflicting reports involves differences in experimental design. Our results suggest that chances of detecting increases in slow inward current are improved by continuous monitoring of the drug effect and by use of appropriate intervals between clamp pulses or stimuli.

We thank T. Colatsky, E. Marban and S. Siegelbaum for comments on the manuscript. The research was supported by grants from the US PHS. R.W. was supported by the Swiss National Science Foundation and R.W.T. is an Established Investigator of the American Heart Association.

R. WEINGART
R. S. KASS
R. W. TSIEN

Department of Physiology,
Yale University School of Medicine,
New Haven, Connecticut 06510

Received 25 November 1977; accepted 28 February 1978.

1. Lee, K. S. & Klaus, W. *Pharmac. Rev.* 23, 193–261 (1971).

2. Fabiato, A. & Fabiato, F. *Eur. J. Cardiol.* **1**, 143–155 (1973).
3. Nayler, W. *Am. J. Physiol.* **225**, 918–924 (1973).
4. Katz, A. M. *J. molec. cell. Cardiol.* **4**, 87–89 (1972).
5. Fozzard, H. A. *Circulation* **47**, 5–7 (1973).
6. Scholz, H. *Naunyn-Schmiedeberg's Arch. Pharmac.* **266**, 445 (1970); in *Recent Advances in Studies on Cardiac Structure and Metabolism 5*, (eds Fleckenstein, A. & Dhalla, N. S.) 35–41 (University Park Press, Baltimore, 1975).
7. Tritthart, H., Weiss, R., Volkman, R. & Späh, F. *Naunyn-Schmiedeberg's Arch. Pharmac.* **282**, R99 (1974).
8. Thyrum, P. T. *J. Pharmac. exp. Ther.* **188**, 166–179 (1974).
9. Greenspan, A. M. & Morad, M. *J. Physiol., Lond.* **253**, 357–384 (1975).
10. McDonald, T. F., Nawrath, H. & Trautwein, W. *Circulation Res.* **37**, 674–682 (1976).
11. Josephson, I. & Sperelakis, N. *J. molec. Cell. Cardiol.* **9**, 409–418 (1977).
12. Dramane, K., Driot, P. & Garnier, D. *J. Physiol., Paris* **63**, 43A (1971).
13. Blood, B. E. *J. Physiol., Lond.* **251**, 69–70P (1975).
14. Cohen, I., Daut, J. & Noble, D. *J. Physiol., Lond.* **260**, 75–103 (1976).
15. Ellis, D. *J. Physiol., Lond.* **273**, 211–240 (1977).
16. Deck, K. A., Kern, R. & Trautwein, W. *Pflügers Arch.* **280**, 50–62 (1964).
17. Weidmann, S. *J. Physiol., Lond.* **127**, 213–224 (1955).
18. Gibbons, W. R. & Fozzard, H. A. *J. gen. Physiol.* **65**, 367–384 (1975).
19. Siegelbaum, S. A., Tsien, R. W. & Kass, R. S. *Nature* **269**, 611–613 (1977).
20. Reiter, M. *Naunyn-Schmiedeberg's Arch. Pharmac.* **242**, 497–507 (1962).
21. Ferrier, G. R. *Circulation Res.* **38**, 156–162 (1976); **41**, 622–629 (1977).
22. Isenberg, G. & Trautwein, W. *Pflügers Arch.* **350**, 41–54 (1974).
23. Reuter, H. *J. Physiol., Lond.* **192**, 479–492 (1967).
24. Vassort, G. *et al.* *Pflügers Arch.* **309**, 70–81 (1969).
25. Tsien, R. W. *Adv. Cyclic Nucleotide Res.* **8**, 363–420 (1977).
26. Gibbons, W. R. & Fozzard, H. A. *Circulation Res.* **28**, 446–460 (1971).
27. Lederer, W. J. & Tsien, R. W. *J. Physiol., Lond.* **263**, 73–100 (1976).
28. Reuter, H. *Prog. Biophys. molec. Biol.* **26**, 1–43 (1973).
29. Kass, R. S., Tsien, R. W. & Weingart, R. *J. Physiol., Lond.* (in the press).
30. Hiraoka, M. & Sano, T. in *Recent Advances in Studies on Cardiac Structure and Metabolism* (ed. Dhalla, N. S.) (in the press).
31. Ferrier, G. R. *Prog. Cardiovascular Dis.* **19**, 459–474 (1977).
32. Kass, R. S., Siegelbaum, S. A. & Tsien, R. W. *J. Physiol., Lond.* **263**, 127–128P (1976).
33. Langer, G. A. & Serena, S. D. *J. molec. cell. Cardiol.* **1**, 65–90 (1970).
34. Bailey, L. & Sures, H. A. *J. Pharm. exp. Ther.* **178**, 259–270 (1971).
35. Besch, H. R. & Schwartz, A. *J. molec. cell. Cardiol.* **1**, 195–199 (1970).

Use of ^3H -muscimol for GABA receptor studies

γ -AMINOBUTYRIC ACID (GABA) is a major transmitter in the mammalian central nervous system (CNS)^{1,2} and studies of synaptic receptors for neurotransmitters have been useful in many areas of neuropharmacology^{3,4}. Although GABA receptors can be studied using ^3H -GABA itself^{5–7}, a ligand which does not bind to GABA uptake sites would be valuable for autoradiography and for other studies of receptor function. Muscimol (3-hydroxy-5-aminomethylisoxazole) is a naturally occurring GABA analogue found in *Amanita muscaria*^{8,9}. It is reported to be a potent agonist at bicuculline-sensitive, strychnine-insensitive postsynaptic receptors of the mammalian CNS^{10–12} and to have minimal affinity for the GABA uptake system¹³. Muscimol also seems to enter the brain after peripheral injection¹⁴ and is aldehyde fixable. I here present evidence of the binding of ^3H -muscimol by brain tissue, comparing binding by membrane preparations *in vitro* with retention of ^3H -muscimol after intravenous administration. The ability of muscimol to alter evoked release of GABA by synaptosomes was also used to verify the ability of muscimol to alter the function of GABA neurones.

Characterisation of ^3H -muscimol binding to membrane preparations from rat brain was carried out with ^3H -muscimol of high specific activity (9.8 Ci mmol⁻¹, New England Nuclear. [$6\text{-}^3\text{H}$]Muscimol was used in these experiments, but side-chain labelled ^3H -muscimol has also been prepared with a somewhat higher specific activity). Rat brains, or various brain regions were treated as described in Fig. 1. Binding of ^3H -muscimol was studied in various sodium-free buffers and optimal binding (highest proportion of specific binding) obtained with use of 40 mM HEPES-Tris buffer, pH 6.8. Addition of 150 mM NaCl to the incubation buffer did not alter specific binding of ^3H -muscimol. Specific binding is that portion of ^3H -muscimol binding which was displaced by an excess (800 nM) of GABA, and was 55–65% of total binding for a final ^3H -muscimol concentration of 2.9 nM.

Figure 1 is a Scatchard plot of the results of the ^3H -muscimol binding studies. When analysed by a nonlinear computer fitting procedure¹⁶, a K_d of 2.7 ± 0.7 nM and a B_{max} of 3.3 ± 0.8 pmol per protein were obtained. If membranes were preincubated in Triton X-100, subsequent

^3H -muscimol binding was enhanced, and Scatchard plots indicated no change in K_d . Similar enhancement could be obtained by preincubation at 37 °C. Both these treatments increased the proportion of specific binding. Table 1a indicates the IC_{50} values for a number of GABA-related drugs and is interesting because it shows stereospecificity in these results: (+)- but not (–)-bicuculline competes with muscimol for binding¹⁷. Note also that picrotoxin, although known to block GABA-mediated inhibition^{18,19}, did not significantly displace bound muscimol. This and other results shown are in general accord with the results of previous ^3H -GABA binding studies^{6,7}.

Table 1b indicates that muscimol and bicuculline can alter the release of ^3H -GABA previously accumulated by rat cortical synaptosomes. These data are most readily explained by postulating that GABA release from GABA terminals is regulated by presynaptic receptors on those terminals and that muscimol stimulates, and bicuculline blocks, those receptors. In this system muscimol seems to be more potent than bicuculline. Many neurones seem to have presynaptic receptors which can regulate the amount of transmitter released^{20–22} and the demonstration that a drug can alter transmitter release appropriately is additional evidence for binding to a receptor site rather than to some other membrane component.

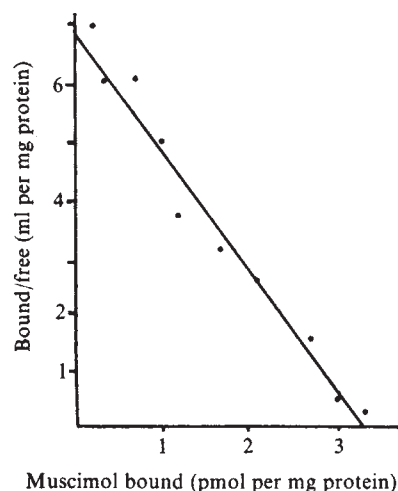


Fig. 1 Binding of ^3H -muscimol to membrane preparations from rat whole brain was studied at 4 °C. The tissues were homogenised in 20 vol 0.32 M sucrose, the P1 pellet¹⁵ collected after a 1,000g centrifugation was discarded and the P2 pellet, collected by a 20-min centrifugation at 20,000g. This pellet was resuspended in water, the suspension frozen overnight and after thawing, the membrane pellet was collected by centrifuging for 30 min at 45,000g. This pellet was washed with 25 mM HEPES-Tris buffer, pH 7.0 and again centrifuged at 45,000g. The pellet was then resuspended in water and could be stored frozen for at least 60 d. Membranes were added to HEPES-Tris buffer, final concentration 40 mM, and various concentrations of ^3H -muscimol were added. Preincubation and incubation times were 10 min. Specific binding was estimated by parallel incubations with an excess of unlabelled muscimol or GABA. Incubations were done at 4 °C in an Eppendorf microfuge tube, with a final volume of 1.05 ml, after adding membranes, any drugs to be studied, buffer, and finally ^3H -muscimol, usually at a final concentration of 2.9 nM. Incubation was carried out inside the microfuge and centrifugation used to terminate the incubation⁵. The pellet obtained after a 12-min centrifugation was rinsed gently with 0.8 ml saline and recentrifuged for 3 min, after which the supernatant was removed and the pellets solubilised by the addition of 300 μl of Protosol (New England Nuclear). The pellets were left for 24 h at room temperature before counting in a liquid scintillation counter. This microfuge technique permits the use of small quantities of tissue and typical assays use 100–200 μg protein per tube. Parallel experiments with 0.5 M Tris-HCl, pH 7.4, or 0.32 M sucrose as the rinse solution gave the same results as the saline rinse, which significantly decreased nonspecific binding.

In other studies, variable concentrations of muscimol were added to Krebs-Ringer HEPES incubation media containing rat cortical synaptosomes and ^3H -GABA (final concentration $0.1\ \mu\text{M}$). After a 5-min preincubation and a 4-min incubation, synaptosomes were collected by Millipore filtration, the filters dried and counted in a liquid scintillation counter. Muscimol concentrations of 10 and $50\ \mu\text{M}$ had no effect on ^3H -GABA uptake, whereas $250\ \mu\text{M}$ and $1\ \text{mM}$ muscimol inhibited GABA uptake by 23.0 ± 4.8 and $41.1 \pm 8.5\%$ respectively ($P < 0.02$, $n = 5$ for all muscimol concentrations studied, $n = 6$ for controls). These results are similar to those obtained in earlier studies with brain slices¹³.

Table 2 presents data from regional studies of brain retention of ^3H -muscimol after peripheral intravenous (i.v.) administration (Table 2a) or *in vitro* binding studies with membrane preparations from the regions indicated (Table 2b) and the results show a generally good correspondence between the two studies. In other studies (unpublished) it was found that the disappearance of ^3H -bicuculline from brain after i.v. administration was slower than that of

Table 1 Inhibitory activity of several GABA-related drugs and muscimol and bicuculline effects on ^3H -GABA release from rat cortical synaptosomes

a IC ₅₀ values for GABA-related drugs*	
Drug	IC ₅₀ (nM)
Muscimol	6
3-Aminopropanesulphonic acid	7
GABA	24
Imidazoleacetic acid	30
β -Guanidinopropionic acid	45
(+)-Bicuculline	1,800
(+)-Narcotine	5,500
(-)-Bicuculline	> 15,000
Picrotoxin	> 15,000
Picrotoxinin	> 15,000
β -Alanine	> 15,000
2,4-Diaminobutyrate	> 15,000
Taurine	> 15,000

b Effects of muscimol and bicuculline on ^3H -GABA release from rat cortical synaptosomes

Condition	Fractional rate constant (min ⁻¹)
KRH medium (8)	0.0072 $\pm$ 0.0010
KRH + 100 μM bicuculline (4)	0.0090 $\pm$ 0.0026
KRH + 1 μM muscimol (4)	0.0075 $\pm$ 0.0015
KRH + 100 μM GABA (4)	0.0168 $\pm$ 0.0031†
High K ⁺ medium (8)	0.0195 $\pm$ 0.0020‡
High K ⁺ + 10 μM bicuculline (5)	0.0230 $\pm$ 0.0034
High K ⁺ + 100 μM bicuculline (5)	0.0329 $\pm$ 0.0054†
High K ⁺ + 250 μM bicuculline (4)	0.0436 $\pm$ 0.0062‡
High K ⁺ + 1 μM muscimol (5)	0.0126 $\pm$ 0.0026‡
High K ⁺ + 25 μM muscimol (5)	0.0087 $\pm$ 0.0031‡
High K ⁺ + 100 μM muscimol (5)	0.0128 $\pm$ 0.0024‡
High K ⁺ + 25 μM muscimol and 100 μM bicuculline (4)	0.0158 $\pm$ 0.0033
High K ⁺ + 100 μM GABA (4)	0.0224 $\pm$ 0.0038

A crude synaptosomal preparation (P2 pellet)¹⁵ was loaded with ^3H -GABA $0.25\ \mu\text{M}$ in Krebs-Ringer HEPES medium for 25 min. Amino-oxyacetic acid (1 mM) was added 5 min after the start of this incubation, which was terminated by adding a large volume of 0.32 M sucrose and then the synaptosomes collected by centrifugation. Synaptosomes equivalent to 10 mg original tissue were collected on Millipore filters²³ and exposed to each experimental medium for 2 min; at the end of a series of media, the radioactivity of tissue and media were measured by scintillation counting and the rate of efflux expressed as a fractional rate constant²⁴. Ion exchange chromatography using Dowex 50 columns indicated that more than 90% of the radioactivity in tissue and media was ^3H -GABA. The increased efflux of ^3H -GABA produced by addition of GABA to the medium has been described previously^{25,26}. The number of experiments is given in parentheses, the rate constants are the mean $\pm$ s.e.m. Significance was estimated by the Kruskal-Wallis nonparametric analysis of variance²⁷.

* The test drugs were studied at three or more different concentrations with ^3H -muscimol at $4.1\ \text{nM}$. The IC₅₀ for specific muscimol binding was estimated by log-probit analysis.

† $P < 0.02$; ‡ $P < 0.01$.

muscimol, suggesting that it dissociates more slowly from GABA receptors. Muscimol injections also reduced the amount of ^3H -bicuculline found in the brain after i.v. injection of bicuculline. The data in Table 2 indicate that much more ^3H -muscimol is bound *in vitro* than is retained by the brain after i.v. administration. The *in vitro* data probably include some 'potential binding sites' not operative *in vivo*, even after correcting for nonspecific binding, which is much less precise in *in vivo* studies.

The brain homogenates used in Table 2a were also used to check for metabolism of ^3H -muscimol. Homogenates were treated with 0.4 N perchloric acid, and the resulting extract loaded onto 5 cm Dowex 50 ion-exchange columns, from which ^3H -muscimol was eluted with 3 M HCl, after first washing the columns with distilled water. The HCl eluate was further studied by thin-layer chromatography on silica gel, using methanol-ammonia 24:1 as the mobile phase. This indicated that 99% of the radioactivity in the eluate had the same R_f as standard muscimol ($R_f = 0.75$) and that 17% of the brain radioactivity did not stick to the resin, 10% of this being volatile (lost when the solution was taken to dryness) and 7% non-volatile (not further characterised at this time). It is possible that the 7% represents a metabolite of muscimol, but similar studies of ^3H -muscimol after *in vitro* binding studies like those described in Fig. 1 indicated no radioactivity other than that accounted for by ^3H -H₂O(THO) and by muscimol itself.

Only a small proportion (0.0002–0.0024) of the injected ^3H -muscimol is retained by the brain after peripheral administration, so autoradiography following i.v. administration of this ligand is not practicable except in small rodents. Studies with steroid hormones²⁸, however, which required similarly high doses, have been very informative. The use of muscimol to treat neurological diseases caused by a loss of GABA neurones has been suggested¹⁷ and such use would involve its peripheral administration. The population of receptors labelled after peripheral administration may differ qualitatively from that accessible to local or intraventricular administration of drug. Ideally, studies of receptor topography should include both systemic and local administration.

Muscimol binding to membrane preparations from rat brain is saturable, rapid in its development (half maximal binding was found in 1-min incubations at 4 °C) and is sodium independent. Since the demonstration of saturable, specific binding to membrane preparations is necessary, but not sufficient, evidence that a drug binds to functioning receptors, the demonstration that muscimol alters the potassium-evoked release of ^3H -GABA from synaptosomes is important. This effect was also antagonised by bicuculline and probably reflects activation of presynaptic GABA receptors. Much higher concentrations of muscimol (250 μM or more) are required to alter synaptosomal GABA uptake significantly than are necessary to decrease evoked release of ^3H -GABA. The concentration of muscimol used (1 μM) is much above the calculated K_d for muscimol binding to brain membranes and studies are now in progress to estimate the effective K_d in the synaptosomal release system. The distribution of bound muscimol 45 min after i.v. injection correlates well with the amount of muscimol bound *in vitro* by membrane preparations from the various brain regions. Muscimol binding *in vivo* and *in vitro* shows the pharmacological characteristics of GABA-receptor binding and so muscimol should be a useful tool in studies of the number, location and kinetic properties of such receptors. It may also interact with presynaptic as well as postsynaptic receptors.

This research was supported by a grant from the US NIH (NS-12368) and a Developmental Neurology programme grant (HD-09704) I thank K. Bilal and A. H. Davis for technical assistance, Dr Richard Young (New England

Table 2 Regional content of ^3H -muscimol after i.v. injection and binding in various brain regions**a** Regional content (fmol per g wet weight) of ^3H -muscimol after i.v. injection

Region	5 min (5)	45 min (4)	75 min (2)	45 min + bicuculline (4)	'Displaceable binding'
Frontal cortex	153 ± 49.7	345 ± 51.1 (0.722)	252	154 ± 34.8	191
Striatum	132 ± 32.6	270 ± 13.5 (0.564)	240	96.8 ± 12.1	173
Hypothalamus	302 ± 98.0	343 ± 25.2 (0.726)	212	154 ± 33.7	189
Substantia nigra	103 ± 65.5	359 ± 60.3 (0.728)	264	120 ± 24.1	239
Thalamus	210 ± 63.5	324 ± 24.0 (0.729)	213	193 ± 60.9	131
Cerebellum	312 ± 88.1	400 ± 32.5 (0.705)	313	135 ± 44.1	265
Pons	219 ± 60.0	328 ± 24.3 (0.669)	257	189 ± 60.0	139
Hippocampus	203 ± 53.0	402 ± 32.8 (0.829)	276	151 ± 66.1	251
Occipital cortex	270 ± 86.8	344 ± 24.4 (0.703)	264	142 ± 51.3	202

b Binding of ^3H -muscimol in various brain regions

	Binding (pmol per g protein)
Cerebellum	455 ± 53
Occipital cortex	311 ± 65
Hippocampus	290 ± 33
Frontal cortex	268 ± 41
Substantia nigra	240 ± 35
Striatum	215 ± 24
Hypothalamus	158 ± 28
Thalamus	115 ± 30
Spinal cord	32 ± 6
Pons	28 ± 4

a, Rats (150–200 g) received ^3H -muscimol ($2.5 \mu\text{g kg}^{-1}$, $600 \mu\text{Ci kg}^{-1}$) by i.v. injection into a tail vein and were decapitated at the times indicated. A blood sample was taken from the neck vessels and the brain dissected on ice. Each brain region was homogenised in pH 7.0 HEPES buffer and an aliquot taken for scintillation counting, with quench correction by internal standardisation. The values given are mean $\pm$ s.e.m. The number given in parentheses for the 45-min group is the mean tissue-plasma ratio for each region, which corrects for any variability in injection technique and efficacy between individual rats. The rats receiving bicuculline and muscimol received bicuculline ($60 \mu\text{g kg}^{-1}$) simultaneously with the ^3H -muscimol. 'Displaceable binding' is the difference between the muscimol bound at 45 min, with and without bicuculline. **b**, Binding studies used frozen membrane preparations as described in Fig. 1, with 10-min incubations and HEPES-Tris buffer, pH 6.8. Nonspecific binding was estimated from parallel incubations containing 800 nM GABA. ^3H -muscimol concentration was 3.3 nM. Each value is the mean of four experiments.

Nuclear) for the interest and perseverance which enabled muscimol to be labelled successfully and Dr J. F. Collins (City of London Polytechnic) for his gift of (–)-bicuculline.

S. ROBERT SNODGRASS

Department of Neurology,
Harvard Medical School
and Department of Neuroscience,
Mental Retardation Research Center,
Children's Hospital Medical Center,
Boston, Massachusetts 02115

Received 5 December 1977; accepted 13 March 1978.

1. Roberts, E., Chase, T. N. & Tower, D. B. (eds) *GABA in Nervous System Function* (Raven, New York, 1976).
2. Iversen, L. L. in *Perspectives in Neuropharmacology* (ed. Snyder, S. H.) 75–111 (Oxford University Press, New York, 1972).
3. Snyder, S. H. *Biochem. Pharmac.* **24**, 1371–1374 (1975).
4. Kahn, C. R. *J. Cell Biol.* **70**, 261–286 (1975).
5. Zukin, S. R., Young, A. B. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4302–4307 (1974).
6. Enna, S. J. & Snyder, S. H. *Brain Res.* **100**, 81–97 (1975).
7. Enna, S. J. & Snyder, S. H. *Molec. Pharmac.* **13**, 442–454 (1977).
8. Muller, G. F. & Eugster, C. H. *Helv. chim. acta* **48**, 910–926 (19.5).
9. Waser, P. G. in *Ethnopharmacologic Search for Psycho-active Drugs* (eds Efron, D., Holmstedt, B. & Kline, N. S.) 419–439 (US Public Health Service Publ. No. 1645, Washington, 1957).
10. Curtis, D. R., Duggan, A. W., Felix, D. & Johnston, G. A. R. *Brain Res.* **32**, 69–95 (1971).
11. Krogsgaard-Larsen, P., Johnson, G. A. R., Curtis, D. R., Game, C. J. A. & McCulloch, R. M. *J. Neurochemistry* **25**, 803–809 (1975).
12. Johnston, G. A. R. in *GABA in Nervous System Function* (eds Roberts, E., Chase, T. N. & Tower, D. B.) 395–411 (Raven, New York, 1976).
13. Johnston, G. A. R. *Psychopharmacologia* **22**, 230–233 (1971).
14. Naik, S. R., Guidotti, A. & Costa, E. *Neuropharmacology* **15**, 479–483 (1976).
15. Gray, E. G. & Whittaker, V. P. *J. Anat.* **96**, 79–88 (1962).
16. Rodbard, D. *Adv. exp. Med. Biol.* **36**, 789–326 (1972).
17. Enna, S. J., Collins, J. F. & Snyder, S. H. *Brain Res.* **124**, 155–190 (1977).
18. Kelly, J. S. & Beurt, P. M. in *Handbook of Psychopharmacology, III* (eds Iversen, L. L., Iversen, S. D. & Snyder, S. H.) 129–188 (Plenum, New York, 1975).
19. Ohata, K. & Highstein, S. M. *Brain Res.* **18**, 538–541 (1970).
20. Starke, K. & Endo, T. *Gen. Pharmac.* **7**, 307–312 (1976).
21. Westfall, T. C. *Physiol. Rev.* **57**, 659–728 (1977).
22. Langer, S. Z. *Biochem. Pharmac.* **23**, 1793–1800 (1974).
23. Levy, W. B., Redburn, D. A. & Cotman, C. W. *Science* **181**, 676–678 (1973).

24. Hopkin, J. & Neal, M. J. *Br. J. Pharmac. Chemother.* **42**, 215–223 (1971).
25. Levi, G. & Raiteri, M. *Nature* **250**, 735–737 (1974).
26. Simon, J. R., Martin, D. L. & Kroll, M. J. *Neurochemistry*, **23**, 981–991 (1974).
27. Conover, W. J. *Practical Nonparametric Statistics* (Wiley, New York, 1971).
28. Gerlach, J. L. & McEwen, B. S. *Science* **175**, 1133–1135 (1972).

Insulin-dependent regulation of the insulin-sensitivity of adipocytes

THE responsiveness of a target organ to insulin is evidently subject to regulation. This phenomenon is best demonstrated by the presence in man and animals of insulin-resistant states^{1–4}. Since these states are usually accompanied by elevations in plasma insulin levels, a role for insulin in the modulation of insulin-sensitivity of target organs has been proposed^{5–8}. Here we present direct evidence for 'self-modulation' by insulin of the insulin-sensitivity of adipocytes. The change in insulin-sensitivity occurs in conjunction with an alteration in the capability of the cells to bind insulin. However, it is not clear if this alteration contributes to the insulin resistance since the change in binding was detected only at relatively high concentrations of ¹²⁵I-labelled insulin.

An *in vitro* organ maintenance system containing pieces of adipose tissue was used to treat adipocytes with insulin in chemically-defined conditions. Epididymal fat pads were removed in sterile conditions from 180-g male Sprague-Dawley rats and cut into small pieces in Parkers medium 199 (refs 7, 8) which contained 0.2 g% (w/v) bovine albumin. The tissue pieces were incubated for 17 h in medium 199 under an atmosphere of 95% O₂–5% CO₂ in the presence or absence of 35×10^{-10} M insulin. The fat pieces were then collected and washed with Krebs–Ringer phosphate buffer, pH 7.4 (ref. 9). Adipocytes were isolated from the fat pieces

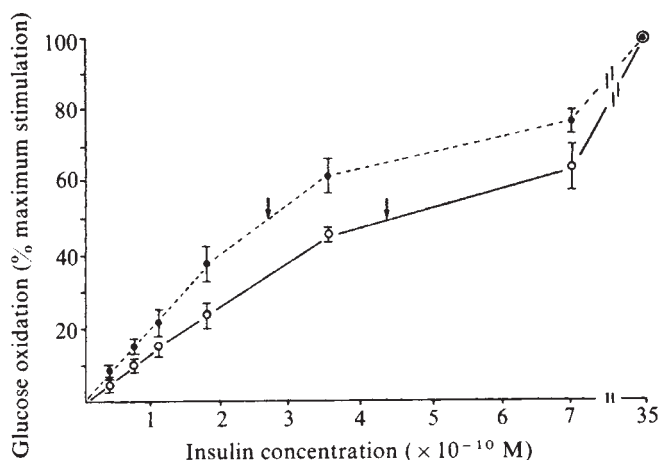


Fig. 1 Insulin enhancement of glucose oxidation in insulin-treated and untreated adipocytes. Pieces of epididymal fat pads were incubated for 17 h in the presence (○) or absence (●) of insulin (35×10^{-10} M). Isolated adipocytes were then prepared and subjected to acute experiments which determined their responsiveness to insulin. Acute experiments involved incubation of isolated cells for 2 h at 37°C with the indicated concentrations of insulin and 0.1 mM U- ^{14}C -D-glucose ($3 \mu\text{Ci } \mu\text{mol}^{-1}$); formation of CO_2 was measured as previously described³. Basal (absence of insulin during the acute experiment) oxidation by insulin-treated and untreated cells were respectively 65.5 ± 9 and 57.1 ± 8 pmol per 2 h per mg lipid (mean $\pm$ s.e.m. of the six experiments). Maximum stimulation of glucose oxidation was produced by 35×10^{-10} M insulin and was 261 ± 31 and 229 ± 31 pmol per 2 h per mg lipid respectively for insulin-treated and untreated cells. The percentage stimulation caused by the various insulin concentrations was calculated by the formula (insulin-stimulated rate minus basal rate) $\div$ (maximal insulin-stimulated rate minus basal rate). Arrows indicate the insulin concentration which causes one-half maximal stimulation of glucose oxidation. The results are the mean $\pm$ s.e.m. of six separate experiments.

by the collagenase method¹⁰ and the cells suspended in the Krebs buffer which contained 3 g% (w/v) bovine albumin. These procedures remove essentially all of the insulin which was present in the insulin-treated fat pieces as indicated by studies using ^{125}I -labelled insulin (data not shown). The concentration of insulin remaining in the incubation medium after the 17-h culture period was variable and ranged from 0.7×10^{-10} to 14×10^{-10} M as measured by radioimmunoassay. After the collagenase step the isolated adipocytes were subjected to acute experiments which tested their responsiveness to insulin. (Maintaining adipose tissue in medium 199 for 17 h in itself changes only one parameter of insulin sensitivity—% maximal stimulation of glucose transport by insulin is decreased. This effect is caused by a 2.5-fold increase in basal transport rate in the absence of a change in the maximum insulin-stimulated rate. The concentration of insulin required to produce half-maximal activation of transport is not altered.)

Figure 1 shows the ability of insulin to enhance glucose oxidation and compares the insulin-responsiveness of cells previously treated with insulin to the hormonal responsiveness of untreated cells. As indicated in Fig. 1 legend, insulin treatment did not change either basal oxidation of glucose (absence of insulin in acute experiments) or maximal oxidation produced by a high insulin concentration. The only difference is in the concentration of insulin required to produce one-half maximal stimulation of glucose oxidation; untreated cells required an insulin concentration of 2.7×10^{-10} M to elicit half maximal stimulation which was significantly less than the amount (4.3×10^{-10} M) required by cells previously exposed to the hormone ($P < 0.05$ as determined by paired t test).

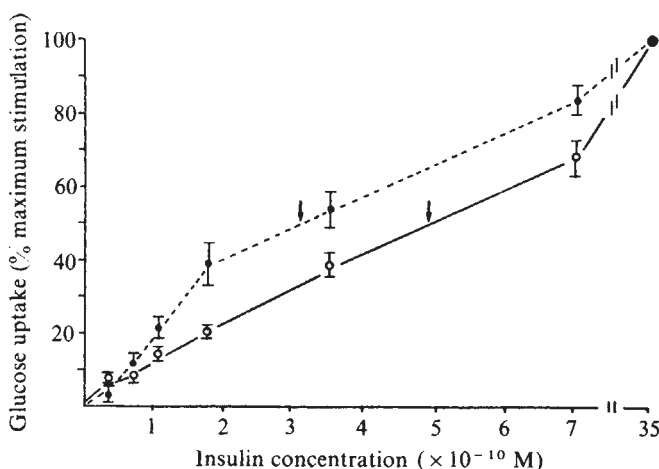
The ability of insulin to stimulate the glucose transport system of these cells was assessed by rapid measurements of

glucose uptake^{9,11}. Again, basal activity and maximal insulin-stimulated activity were not changed by prior treatment with the hormone (Fig. 2 legend). In agreement with the glucose oxidation studies, the insulin-treated cells were less responsive to the hormone as shown by the insulin dose-response studies (Fig. 2). The concentrations of insulin required to produce half-maximal activation of glucose transport for insulin-treated and untreated cells were respectively 4.8×10^{-10} and 3.1×10^{-10} M; this difference was significant as indicated by paired t analysis ($P < 0.02$). We obtained similar results when the hexose transport activity of treated and untreated cells was estimated by measuring the uptake of 2-deoxyglucose, a sugar which is phosphorylated but not metabolised further¹¹ (data not shown).

These studies are the first direct demonstration of insulin regulation of the insulin-sensitivity of a target cell. The insulin-dependent change in sensitivity is mediated through a shift in the dose-response curve to insulin and not by any obvious changes in either the glucose transport system or intracellular glucose metabolism. Since the rate of glucose oxidation is dependent on the rate of glucose transport, the alteration responsible for the insensitivity to insulin is limited to the insulin receptor and/or the mechanism responsible for coupling the receptor to the glucose transport system.

The insulin receptor content of the adipocytes was estimated by measurements of the specific binding of ^{125}I -labelled insulin^{3,9}. Figure 3 shows a Scatchard analysis of the insulin binding data; the insulin-treated cells tend to bind less iodinated insulin than the control cells at the high affinity portion of the curves (^{125}I -labelled insulin concentrations of 5×10^{-11} to 5×10^{-10} M) but this difference was not statistically significant. Maximum high affinity binding was $0.34 \text{ fmol per mg lipid}$ for control cells compared with $0.25 \text{ fmol per mg lipid}$ for insulin-treated cells. Also, the dissociation constant (K_d) for the high affinity portion of the binding curves was the same (6×10^{-10} M) for both types of cells. In addition, the ability of the cells to degrade insulin was not altered by the 17-h insulin treatment. At a ^{125}I -

Fig. 2 Insulin enhancement of glucose uptake by insulin-treated (○) and untreated (●) cells. Pieces of adipose tissue were treated for 17 h with insulin followed by an isolation of adipocytes as described in Fig. 1. During the acute experiments, the adipocytes were preincubated with the indicated concentrations of insulin for 30 min at 37°C . The rate of glucose uptake was then measured using a 30-s assay^{9,11}. Basal rates of glucose uptake for insulin-treated and untreated cells were respectively 2.8 ± 0.5 and 3.6 ± 0.7 pmol per 30 s per mg lipid (mean $\pm$ s.e.m. of seven experiments). Maximal rates of glucose uptake caused by a 35×10^{-10} M concentration of insulin were 11.1 ± 1 and 11.6 ± 1 pmol per 30 s per mg lipid respectively for treated and untreated cells. The percentage maximum stimulation produced by the various insulin concentrations was calculated as described in Fig. 1. The results are the mean $\pm$ s.e.m. of seven separate experiments.



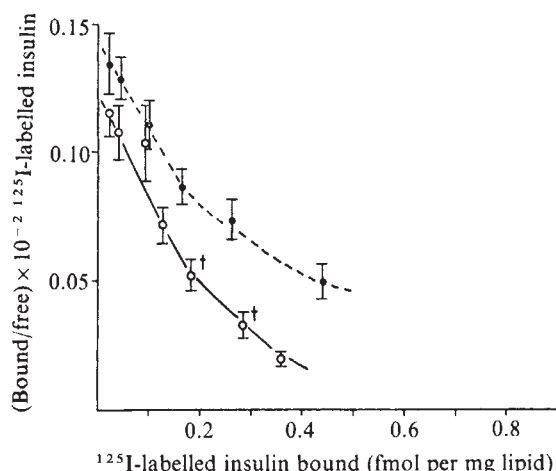


Fig. 3 Scatchard analysis of the specific binding of ^{125}I -labelled insulin to insulin-treated ($\circ$) and untreated ($\bullet$) fat cells. Adipocytes were treated with insulin and isolated from the fat pieces as for previous experiments. Specific binding of ^{125}I -labelled insulin to the isolated cells was as detailed elsewhere^{3,9}. Results are the mean $\pm$ s.e.m. of seven separate experiments. $\dagger$, $P < 0.05$ (paired t test).

insulin concentration of 5×10^{-10} M, both groups of cells degraded approximately 5% of the iodohormone in the conditions used to measure insulin binding^{3,9}.

Only with larger concentrations of ^{125}I -labelled insulin did the difference in insulin-binding become significant. This difference is evident in the low affinity portion of the Scatchard plot (^{125}I -labelled insulin concentrations of 1×10^{-9} to 5×10^{-9} M). The K_d for the insulin-treated cells was slightly lower (1.3×10^{-9} M) than that (2.6×10^{-9} M) of the control cells. Moreover, the total amount of low affinity binding is clearly less in insulin-treated cells.

The insulin-dependent changes in low affinity insulin binding sites may or may not be involved in the alteration(s) which cause the insensitivity to insulin. The changes in insulin sensitivity of the cells are apparent only at insulin concentrations where the amount of insulin binding is not significantly altered (high affinity binding). However, the difference in low affinity insulin binding may reflect subtle alterations in the insulin receptor that in some manner contributes to the hormonal resistance of the cell.

Gavin *et al.*¹² demonstrated an insulin-dependent reduction in insulin binding sites on cultured lymphocytes following incubation for 16 h with high concentrations of insulin (10^{-8} M or greater). However, when lower levels of insulin were used (10^{-9} M), no change in binding was detected. The effect of the alteration in insulin binding on insulin-sensitivity could not be determined since these cells have no known responses to the hormone¹³.

Our results show that a high physiological concentration of insulin changes the insulin-responsiveness of a target cell. The location(s) of the alteration(s) responsible for this change may reside in the insulin receptor or in post-receptor events but probably not in the effector systems (glucose transport and glucose metabolism). Using polyacrylamide gel electrophoresis, we have shown the existence of two distinct insulin binding components on fat cell plasma membranes, one of which is converted to a low affinity species by physiological concentrations of insulin (M. Krupp and J. L., in preparation). Thus, insulin treatment of fat cells may induce a change in the insulin receptor which is difficult to detect by conventional insulin-binding techniques. It remains unclear whether the insulin-binding capability of a cell determines its sensitivity to insulin or whether its responsiveness is modulated by changes in the immediate events activated by insulin-receptor association. Further characterisation of the insulin binding site(s) and the process

which couples the insulin receptor to an effector system (for example, glucose transport) is needed before these questions can be answered.

We thank Dr John Amatruda for useful criticism and Dr Paul Woolf for assistance in radioimmunoassays. This work was supported by grants from the Juvenile Diabetes Foundation, Weight Watchers Foundation and US NIH grant AM-20129.

JAMES N. LIVINGSTON
BARBARA J. PURVIS
DEAN H. LOCKWOOD

Endocrine-Metabolism Unit,
Department of Medicine,
University of Rochester Medical Center,
601 Elmwood Avenue, Rochester, New York 14642

Received 16 December 1977; accepted 3 April 1978.

1. Roth, J. *Metabolism* **22**, 1059–1073 (1974).
2. Lockwood, D. H., Livingston, J. N. & Amatruda, J. M. *Fedn Proc.* **34**, 1564–1569 (1975).
3. Livingston, J. N., Cuatrecasas, P. & Lockwood, D. H. *Science* **177**, 626–628 (1972).
4. Olefsky, J. M. *Diabetes* **25**, 1154–1162 (1976).
5. Rabinowitz, D. A. *Rev. Med.* **21**, 241–258 (1970).
6. Grey, W. & Kipnis, D. M. *New Engl. J. Med.* **285**, 827–831 (1971).
7. Smith, U. J. *clin. Invest.* **53**, 91–98 (1974).
8. Bernstein, R. in *Regulation of Adipose Tissue Mass* (eds Vague, J. & Boyer, J.) 122–128 (Excerpta Medica, Amsterdam, 1974).
9. Livingston, J. N. & Lockwood, D. H. *J. biol. Chem.* **250**, 8353–8369 (1975).
10. Rodbell, M. J. *J. biol. Chem.* **239**, 375–380 (1964).
11. Livingston, J. N. & Lockwood, D. H. *Biochem. biophys. Res. Commun.* **61**, 989–996 (1974).
12. Gavin, J. R., III, Roth, J., Neville, D. M., Jr, DeMeys, P. & Buell, D. N. *Proc. natn. Acad. Sci. U.S.A.* **71**, 84–88 (1974).
13. DeMeys, P. in *Methods in Receptor Research* (ed. Blecher, M.) 315 (Marcel Dekker, New York, 1976).

Transient kinetics of sarcoplasmic reticulum $\text{Ca}^{2+} + \text{Mg}^{2+}$ ATPase studied by fluorescence

IN the sarcoplasmic reticulum (SR) of skeletal muscles ion transport involves an ATP driven calcium pump. The mechanism by which calcium ions are transported across the membrane is unknown. It is reasonable to assume, however, that some conformational changes in the transport protein are associated with this process. These changes might be more subtle than a complete rotation or translation of the protein¹. The experiments described here have been designed to follow the modification of the protein conformation by measuring changes in strength of the protein's tryptophan fluorescence. We have observed transient changes of intensity induced by calcium ions and fluorescence fluctuations during one single pumping cycle of the enzyme. The kinetic pathway of the ATPase reaction which is linked to the ionic transport has been investigated previously by fast techniques, such as chemical quench-flow and fluorescence with calcium indicators^{2,3}. We have attempted to relate the conformational events observed to some steps of the proposed enzymatic schemes^{2–5}.

We have shown previously that binding of calcium ions to the high affinity sites modifies the intrinsic fluorescence of the calcium pump protein⁶. A complete concentration dependence of the amplitude of the response was obtained and compared with the activation of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ ATPase. Taken together, these results strongly suggest that the calcium ions induce a conformational change in the protein which is responsible for the activation of the ATPase activity. Using stopped-flow fluorimetry we followed the kinetics of the fluorescence change induced by calcium binding or release in the absence of ATP (Fig. 1). The rate of the signal rise after binding is relatively slow (5 s^{-1} at 22°C) even at saturating calcium concentrations ($>10 \mu\text{M}$), and certainly does not represent directly the binding of calcium since it gives a too low second-order rate constant. A concentration dependence of the rate was obtained which shows a nonlinear relationship between the rate constant and the calcium concentration. The experi-

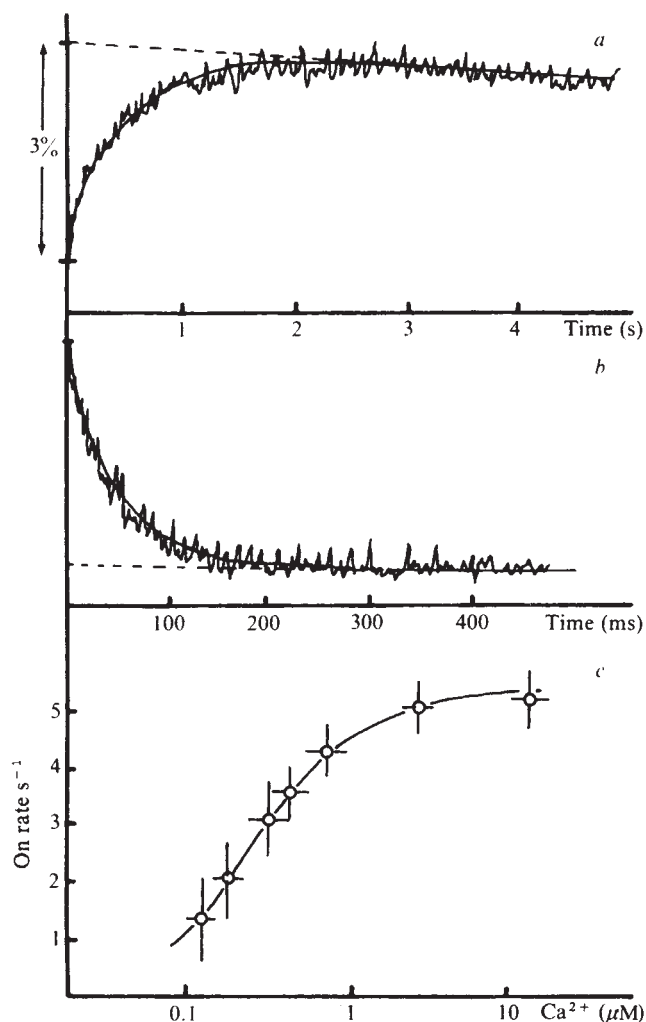
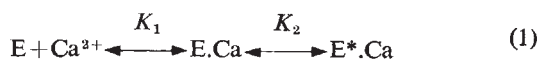


Fig. 1 Stopped-flow record of the fluorescence changes associated with the binding (a) or the release (b) of calcium ions. These experiments were performed at 11 °C with a Durrum stop-flow fluorimeter. Excitation wavelength was 296 nm and the emitted light was filtered through a cut-off filter WG 335. The medium contained 20 mM Tris-maleate, 80 mM KCl and 5 mM Mg²⁺. Other reagents were: a, Syringe 1, sarcoplasmic reticulum vesicles (0.15 mg protein per ml) and 50 μM EGTA. Syringe 2, 100 μM Ca²⁺. b, Syringe 1 (0.15 mg protein per ml) and 20 μM Ca²⁺. Syringe 2, 100 μM EGTA. The line drawn under the trace is a fit by a single exponential. c, Calcium concentration dependence of the on-rate at 22 °C with varying calcium-EGTA ratio. The free calcium concentration was calculated as described in ref. 6. If in a two step process like $E + Ca \xrightleftharpoons{K_1} E.Ca \xrightleftharpoons{K_2} E^*.Ca$, $E.Ca$ equilibrates much faster than $E^*.Ca$, then the rate of appearance of $E^*.Ca$ can be written $k_{app} = k_2^- + k_2^+ / (1 + 1/(K_1 \times Ca^{2+}))$. The data are well fitted with $K_1 = 3 \times 10^6$ M⁻¹, $k_2^+ = 5.3$ s⁻¹ and $k_2^- = 0$ (higher limit $k_2^- = 1$ s⁻¹).

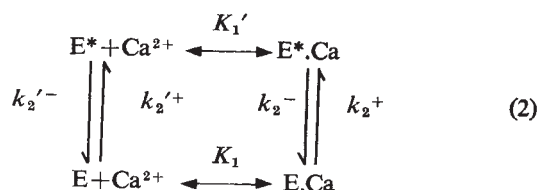
mental data are well fitted by an hyperbolic function, confirming a two-step process with a fast binding followed by a slow isomerisation:



The state E^* has a fluorescence strength $\sim 3.5\%$ higher than that of E .

In fact, as described in Fig. 1 the rate constant k_2^- must be rather small: < 0.5 – 1 s⁻¹. This value is much lower than the rate obtained when the reaction is reversed by the removal of the bound calcium in an excess EGTA. The fluorescence drop which is then observed proceeds at a rate of about 30 s⁻¹

at 22 °C. Hence a second route has to be taken into account and the complete process may be written:



With: $K_1 = 3 \times 10^6$ M⁻¹, $k_2^+ = 5$ s⁻¹, $k_2^- \leq 0.5$ – 1 s⁻¹ and $k_2'^- = 30$ s⁻¹. Since the E state is the major species present without calcium the equilibrium constant K_2' has to be small. Taking $K_2' \leq 10^{-1}$ and $K_2 \sim 5$ gives a binding constant K_1' in the range of 10^5 M⁻¹ which is fairly large. The involvement of the second pathway to the calcium binding is dependent on the value taken for $k_2'^+$, this contribution should be rather small, however, as the shape of the curve of the calcium concentration dependence of the on-rate is well reproduced by the process (1). The temperature dependences of the rates have been investigated down to -5 °C (in a glycerol-water mixture below 0 °C). A very remarkable feature of the Arrhenius plots is the very important reduction of the rates of the fluorescence changes below -2 °C (Fig. 2). From about 12 kcal mol⁻¹ the activation energy of the conformational change becomes of the order of 40 kcal mol⁻¹. There is the possibility that the break in the plot is due to an unidentified rate limiting step observable at low temperature, but the high activation energy suggests a phase change occurring in the lipid matrix greatly increasing the energy required for the conformational change. From previous studies there is no clear evidence of a global phase change in the sarcoplasmic reticulum membrane above 0 °C (ref. 7). We cannot exclude the possibility that this might occur at sub-zero temperatures or that the change is limited to lipids bound to, or surrounding, the hydrophobic regions of the pump protein.

Adding ATP to the enzyme in the $Ca.E^*$ state initiates a turn-over of the enzyme and a transient fluorescence change is observed (Fig. 3) which certainly corresponds to the conformational changes associated with the ion translocation. In order to record one single turn-over the amount of ATP added is equal or slightly lower than the molar concentration of the ATP high affinity sites (3.3 – 3.8 nmol mg⁻¹) (ref. 8). Below -3 °C the transient fluorescence change presents two phases with first a fall in intensity followed by a slower rise where the fluorescence returns to the initial level. This event can be repeated several times and if the ATP concentration after injection exceeds the sites concentration a longer period of low fluorescence is observed whose duration is proportional to the amount of ATP added.

The generally accepted scheme is that the transport proceeds through the formation of a phosphoenzyme after the fast release of ADP in the medium⁵. The number of phosphorylated intermediates with distinct chemical properties²⁻⁴ will be discussed elsewhere, but the rate of phosphoenzyme formation and decay are two important parameters which enable us to assign enzymatic stages to the different phases of the events observed during the turn-over. Therefore we have repeated the experiments in the same temperature conditions and measured the phosphoenzyme concentration during one single turn-over. Using fast filtration techniques (described in Fig. 2) we have observed that, as expected, ¹⁴C-ADP was released into the medium in a time which is much shorter than the dead time of the method (about 4 s), while an experiment using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ indicates that the phosphate remains bound to the enzyme. The phosphoenzyme decays at a rate of about 0.07 s⁻¹ at -4 °C (Fig. 2). The release of inorganic phosphate is accompanied by H⁺ production; measurement with a glass electrode yields the same rate constant.

The event shown in Fig. 3 is well reproduced by two succeeding transitions with rate constants $k_a = 0.07$ s⁻¹ and $k_b = 0.04$ s⁻¹. The rates of the first steps of the reaction, namely

ATP binding, phosphoenzyme formation and ADP release, are too fast to account for the first fluorescence drop, and this is more likely related to the phosphoenzyme decay which proceeds at the same rate. In fact it is probable that calcium is released in the internal medium at the same time and the transient state of low fluorescence might then represent the enzyme free of calcium. The amplitude calculated for the fluorescence of the intermediate state (-3%) gives additional support for this

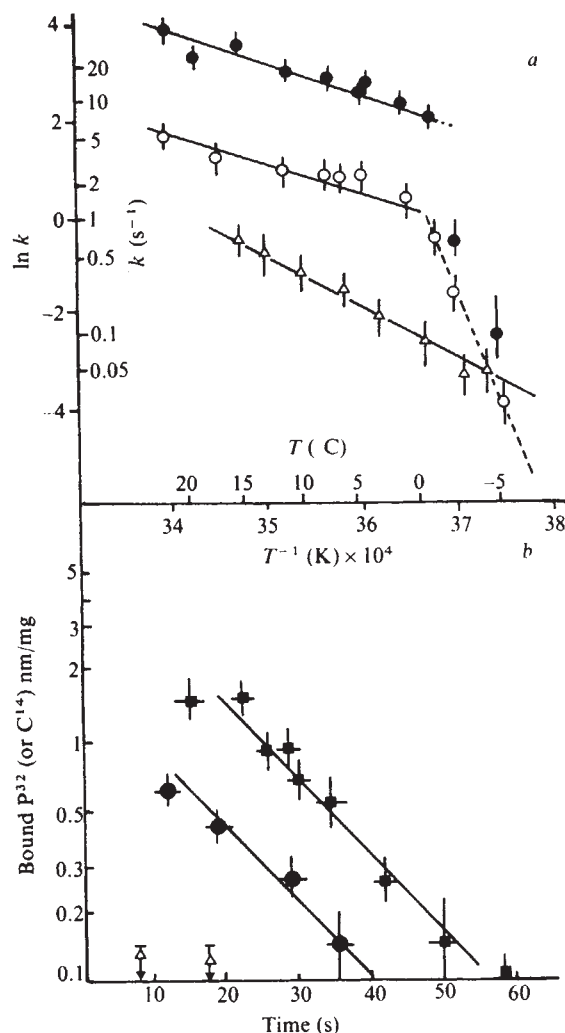


Fig. 2 *a*, Arrhenius plot of the rate of the fluorescence change associated with the binding ($\circ$) and release ($\bullet$) of the calcium ions. The points below 0°C were obtained with a Hitachi MPF-2A spectrofluorimeter in a cooled stirred cuvette⁶ and with a 20% glycerol-buffer mixture. Some points above 0°C were recorded using the stop-flow apparatus with and without glycerol, there was no observable effect of glycerol on the rates. Below 0°C the pH of the solutions were measured by comparison with phosphate buffer containing 20% glycerol. The pH dependence of the rate of fluorescence changes was weak and were checked from pH 6.8 to 7.7. Data (Δ) correspond to the measurement of the rate of H^+ production associated with inorganic phosphate release during one turn-over of the enzyme. Conditions were: 0.1 mM Tris-maleate (pH 7.2), 80 mM KCl, 5 mM Mg^{2+} , 0.75 mg ml^{-1} membrane proteins (about $2.5\text{ }\mu\text{M}$ for the ATP sites) and $50\text{ }\mu\text{M}$ Ca^{2+} . ATP was added to a final concentration of $1.2\text{--}2.5\text{ }\mu\text{M}$. pH was recorded by a PHM64 radiometer pH meter. *b*, Measurement of phosphorylated enzyme by fast filtration at -4°C . At zero time 0.5 to $1\text{ }\mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP was injected into 2 ml of buffer containing 20 mM Tris-maleate, 80 mM KCl, 5 mM Mg^{2+} , $50\text{ }\mu\text{M}$ Ca^{2+} , 0.5 mg sarcoplasmic reticulum vesicles ($\sim 1\text{ }\mu\text{M}$) and 20% glycerol. $\blacksquare$ $1\text{ }\mu\text{M}$ and $\bullet$, $0.5\text{ }\mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP. After mixing the solution is filtered through a Millipore filter. The time indicated is the incubation plus filtration time. A blank is made by filtering the same solution without S.R. vesicles. An analogous method was described previously⁸. Data marked (Δ) were obtained using the same technique except that the substrate was replaced by $1\text{ }\mu\text{M}$ ^{14}C -ATP.

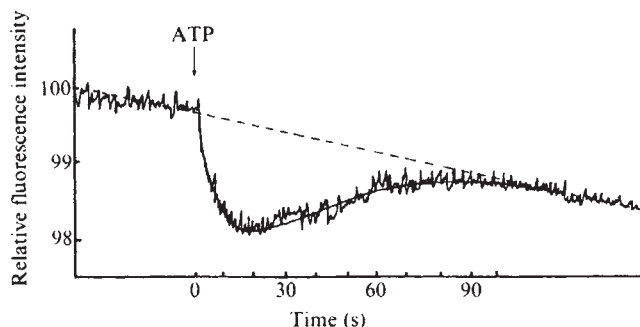
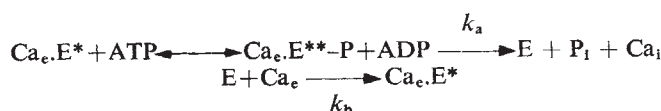


Fig. 3 Single turn-over experiment at -4°C recorded in the fluorimeter. Enzyme concentration, $90\text{ }\mu\text{g ml}^{-1}$ (about 300 nM) in the presence of $50\text{ }\mu\text{M}$ calcium. Final ATP concentration after injection, 250 nM . The smooth trace is from a calculation of the process:

$A \xrightarrow{k_a} B \xrightarrow{k_b} A$, with $k_a = 0.07\text{ s}^{-1}$, $k_b = 0.04\text{ s}^{-1}$ and the B state having a fluorescence 3% lower than the A state. The dashed line corresponds to the photolysis rate.

hypothesis. According to this scheme the final rise is attributed to the external calcium re-binding; again the rate constant obtained for this phase is identical to that found for calcium binding (Fig. 2). This sequence of events can be written:



Above 0°C the dephosphorylation becomes much slower than the re-binding (Fig. 2), and the fluorescence change observed during one turn-over becomes weaker. With a molar excess of ATP the phosphoenzyme accumulates and after the injection of ATP one observes a decrease in fluorescence of 0.5 to 1% . This is why the state $\text{Ca}_e\text{E}^{**}\text{-P}$ is marked with a double asterisk since it is probably a stage of slightly different fluorescence from Ca_eE^* .

The present resolving power of the experiment does not allow a more precise identification of the transition (a), although we can say that the conformational change occurs at the same rate as the dephosphorylation. However, as suggested by several authors^{3,4}, the true translocation step probably occurs between two different phosphorylated intermediates. If this is true, it is likely that the transition between these two states is the true location of the conformational change observed in fluorescence studies and, at least at the temperature of our experiment, this transition is then limiting the rate of dephosphorylation.

If the above identification of events is correct, the other major conformational change of the pump protein during one pumping cycle would be the re-set of the enzyme produced by the re-binding of calcium ions.

YVES DUPONT

Laboratoire de Biophysique
Moléculaire et Cellulaire (E.R. 199),
Département de Recherche Fondamentale,
Centre d'Etudes Nucléaires de Grenoble
85X, 38041 Grenoble Cedex, France

JOHN BARRINGTON LEIGH*

Max Planck Institut für Medizinische Forschung,
Abteilung Biophysik,
69 Heidelberg 1, FRG

Received 28 September 1977; accepted 6 March 1978.
*Present address: Department of Immunology, Faculty of Medicine,
University of Alberta, Edmonton, Alberta, Canada.

- Dutton, A., Rees, E. D. & Singer, S. J. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1532-1536 (1976).
- Froehlich, J. P. & Taylor, E. N. *J. biol. Chem.* **250**, 2013-2021 (1975).
- Ikemoto, N. *J. biol. Chem.* **251**, 7275-7277 (1976).
- Carvalho, M. G. C., de Souza, D. G. & de Meis, L. *J. biol. Chem.* **251**, 3629-3636 (1976).
- Hasselbach, W. *Enzyme* **10**, 431-467 (1975).
- Dupont, Y. *Biochem. biophys. Res. Commun.* **71**, 544-550 (1976).
- Davies, D. G., Inesi, G. & Gulik-Krzywicki, R. *Biochemistry* **15**, 1271-1276 (1976).
- Dupont, Y. *Eur. J. Biochem.* **72**, 185-190 (1977).

The number of genes in *Drosophila melanogaster*

THE number of genes in an organism is one of its fundamental biological parameters and relates to the number of functions required to construct that organism and determine its physiological characteristics. It is generally assumed that with increasing organic complexity the number of genes of the species must increase. The possibility does exist, however, that increasing complexity results from the effects of interactions between a small and relatively constant number of genes. The results presented here support the idea that the number of genes of the insect *Drosophila* is of the order of 5,000 and therefore not very different from the number of genes estimated for bacteria.

In *Drosophila* two approaches, the genetic and the molecular, have been used to estimate this number. The former depends on the occurrence of mutant phenotypes (for example, lethals, visibles, enzymatic, steriles, behavioural). In making this estimate, genes are defined as complementation groups that can be mapped linearly as individual loci by recombination. In *Drosophila* it is possible to relate those maps to physical distances in the giant salivary chromosomes. For small chromosome intervals it has been found that in this organism there is a numerical relationship of about 1 between the number of complementation groups and the number of bands¹⁻³. If this finding can be generalised to the entire *Drosophila* genome, the 5,000 bands of its giant chromosomes may correspond to as many genetic loci. This is probably an underestimate because it might not be possible to score the phenotype of a fraction of the possible mutations. This could be because there is too much remnant wild-type activity; or a low mutation rate of individual loci for a given mutagen; or because of the existence of alternative pathways; or for other reasons such as gene repeats, redundant regions and operon-like genetic systems. That fraction is, however, probably low because in chromosome intervals, on which extensive mutagenesis experiments have been carried out, particular bands may contain one or, occasionally two, but rarely zero complementation groups^{1,4,5}.

The fraction of the *Drosophila* genome containing unique DNA sequences of sufficient length to code for average sized proteins, suggests that the number of genes would be more than 80,000 (ref. 5). The average chromosome band contains about 20,000 base pairs, adequate for about 20 protein-coding genes⁴⁻⁶. The function of this excess of DNA relative to the number of complementation groups is unknown, but it could include genes not detected by standard mutagenesis experiments, or indicate the existence of redundant regulatory regions⁷. An estimate based on the number of different mRNA sequences present in either developing or adult *Drosophila*, suggests that this number is about 5,000 (ref. 8). Again, this could be an underestimate if some mRNAs (for regulatory proteins, for example) are in concentrations below the resolution of the method, or if gene regulation is mediated by nuclear RNAs instead of by proteins⁷.

Here we are evaluating the phenotype of entire chromosome deficiencies as opposed to point mutants. We thus overcome the shortcomings of mutational analysis and can define the effects of the removal of whatever coding regions—structural or regulatory—are present in these DNA intervals. Lethality is easy and unequivocal to score; about 90% of the complementation groups are capable of mutating to a lethal condition¹⁻³, so chromosome deficiencies are lethal in homozygous individuals. Lethal mutations can be studied in cells following mitotic recombination in heterozygous individuals⁹. Of 86 zygotic lethal point mutations in the X chromosome, studied in this way, only 9% were cell lethal in epidermal cells of the tergites^{10,11}. Since some of the cell-viable mutants studied might be leaky (incomplete) mutations of cell-lethal loci, the pro-

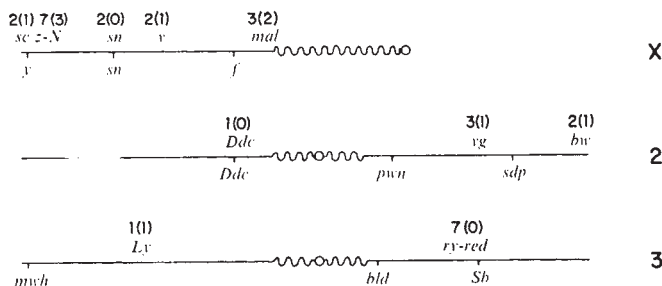


Fig. 1 Location of the studied deficiencies (above) and cell markers in the chromosomes (X, 2 and 3). Number of deficiencies studied in the different intervals; numbers in parentheses are of those viable in clones. Intervals: *sc*, scute; *z-N*, zeste-Notch; *sn*, singed; *v*, vermilion; *mal*, maroon-like; *Ddc*, Dopadecarboxylase; *vg*, vestigial; *bw*, brown; *Ly*, Lyra, *ry-red*, rosy-red. Cell markers: *y*, yellow; *sn*, singed; *f*, forked; *Ddc*, Dopadecarboxylase; *pwn*, pawn; *sdp*, sandpaper; *mwh*, multiple wing hairs; *bld*, bald; *Sb*, Stubble. (See refs 12, 13 and 16 for further descriptions.)

portion of cell lethals among zygotic lethals may be higher.

We studied the viability of 30 deficiencies, lacking 2–30 chromosome bands, located in different regions of the five major chromosome arms (Fig. 1). The different deficiencies were *cis*-coupled with cell-marker mutations and crossed to flies carrying control cell-marker mutants either in the same or in another chromosome arm^{12,13}. Homozygous clones were generated in the tergites by mitotic recombination induced by X rays (1,000 R), applied in the larval period. They show the control cell-marker phenotype with expected frequency and clone size^{12,13}. The presence of the cell marker that labels the homozygosis for the deficiency in normal frequency and clone size is indicative of its viability. Ten deficiencies were found to be viable in clones in the tergites. Table 1 shows the relationships between the number of bands lacking in the deficiency and viability. Viable deficiencies are of different sizes, the two largest ones lacked 14 bands (*Df*(1)260-1) and 28 bands (*Df*(2R)vg⁸). Six of the deficiencies correspond to the X-chromosome region studied by Judd *et al.*¹. From these, three deficiencies contained complementation groups with lethals not viable in male tissue in gynandromorphs, and three (*Df*(1)65j26), *Df*(1)64j4 and *Df*(1)64f1) contained only lethals which were viable in gynandromorphs¹⁴. Of these latter three, one, *Df*(1)65j26), was viable in clones of epidermal cells. This suggests that there exists in the other two regions at least one cell-viability locus not previously detected in mutagenesis experiments or, alternatively, that its lethality results from additive effects of cell-viable loci. The fact that large deficiencies (more than ten bands) are homozygous viable suggests that additive effects are not major causes of cell lethality.

If sequences coding for cell viability are scattered randomly throughout the genome, the average size of viable deficiencies will reflect the ratio of cell-viable to cell-lethal genes. The average number of bands of viable deficiencies found in this work was 8.3, which is close to that of the 9% found for the frequency of cell lethals among point-mutant lethals. Thus the

Table 1 Relationship between the number of chromosome bands lacking in different deficiencies and their viability in clones of homozygous deficient cells

	2–4	5–10	11–20	20–30
No. of deficient chromosome bands				
Deficiencies	9	11	5	5
Cell viable	4	3	2	1

ratio, for point mutants, of cell lethals to lethals is similar to the ratio of cell lethals to chromosome band deficiencies. It must be pointed out that this evaluation does not directly tell us the number of mutable loci present in the genome; we depend for that on mutagenesis experiments. The results do indicate, however, that for functions required for cell viability, chromosome intervals do not contain many more coding sequences (structural or regulatory) than those that could be detected by standard mutagenesis experiments. Those functions which in null-alleles have no scorable phenotype in flies¹⁵, because of gene repeats or physiological homeostasis, cannot be detected in clones. The fraction of such genes and, if this is large, their biological significance, remains to be evaluated.

We thank Miss Ana C. Andreu for technical assistance. This work was supported by the Comision Permanente del Descuento Complementario and CAIT grant 1529.

A. GARCIA-BELLIDO
P. RIPOLL

Centro de Biología Molecular,
CSIC, Universidad Autónoma,
Madrid-34, Spain

Received 22 November 1977; accepted 21 March 1978.

- Judd, B. H., Shen, M. W. & Kaufman, T. C. *Genetics* **71**, 139 (1972).
- Hochman, B. *Genetics* **67**, 235 (1971).
- Lifschytz, F. & Falk, R. *Mutat. Res.* **6**, 235 (1968).
- Beermann, W. in *Results and Problems in Cell Differentiation* **4**, 1-33 (eds Beermann, W. & Springer, 1972).
- Laird, C. D. *A. Rev. Genet.* **7**, 177 (1973).
- Lefevre, G. Jr. *A. Rev. Genet.* **8**, 51 (1974).
- Davidson, E. H. & Britten, R. J. *Q. Rev. Biol.* **48**, 565 (1973).
- Bishop, J. O. et al. *Phil. Trans. R. Soc. B* **272**, 147 (1975).
- Demerec, M. *Proc. natn. Acad. Sci. U.S.A.* **22**, 350 (1936).
- Ripoll, P. & García-Bellido, A. *Nature new Biol.* **241**, 15 (1973).
- Ripoll, P. *Genetics* **86**, 357 (1977).
- García-Bellido, A. *Molec. gen. Genet.* **115**, 54 (1972).
- García-Bellido, A. & Dapena, X. *Molec. gen. Genet.* **128**, 117 (1974).
- Shannon, M. P., Kaufman, T. C., Shen, W. M. & Judd, B. H. *Genetics* **72**, 615 (1972).
- O'Brien, S. J. *Nature new Biol.* **242**, 52 (1973).
- Lindsley, D. L. & Grell, E. H. *Carnegie Inst. Wash. Publ. No.* 627 (1968).

Unusual genes at the aminoterminal of human immunoglobulin variants

HEAVY chain disease proteins¹⁻³ are defective immunoglobulin molecules caused by a mutation of heavy chain structural genes. Defective γ , α , and μ chains have been described in man, and a few abnormal myeloma proteins have been identified in the mouse⁴. In general the variants are shorter than their normal counterparts because of terminal or internal deletions. The deletions can affect either the constant (C) genes, or both the C and variable (V) genes. When the mutation affects C genes, the defect seems not to be random, as it generally involves either intact domains and/or interdomain regions⁵. On the other hand, V gene deletions seem not only to be random in location and size, but in some instances to have distinct features such as unusual amino acid sequences and/or the presence of carbohydrate moieties in unusual sites⁶. We present here studies of the aminoterminal regions of two new heavy chain disease proteins (HAR and LEA)⁶ and more quantitative data on certain ambiguities of a third protein (CRA) which has been partially sequenced⁵. Table 1 lists some physicochemical properties (details will be published elsewhere) of heavy chain disease proteins LEA, HAR and CRA.

To isolate the blocked aminoterminal region, pronase digests (in 0.1M NH_4HCO_3 , pH 8.2 for 2 h at 37° C at an enzyme ratio of 1:50 (w/w)) of completely reduced and alkylated HAR and LEA proteins were passed through a Dowex AG 50 W-X2 column, and the eluted peptides were purified by paper electrophoresis⁷ at pH 6.5. Their amino acid compositions and postulated sequences are shown in Table 2. In the case of protein LEA, two peptides were obtained: Glu, Leu and Glu₂, Ser, Gly, Val, Leu. The amino acid sequence of the NH₂ terminus of the third protein CRA which is unblocked is also shown in Fig. 1. At step 3, Leu and Ile were recovered in equal

Table 1 Molecular weight and purity of protein samples were determined by sodium dodecyl sulphate polyacrylamide disc electrophoresis under reducing conditions¹⁴

Mutant	Molecular weight	Subclass of H chain	NH ₂ Terminal
LEA (ref. 6)	45,000	γ l	Blocked
HAR	34,000	γ l	Blocked
CRA (ref. 5)	25,000	γ l	Gly

Amino terminal residues were identified by the dansyl method¹⁵ on thin layer polyamide plates¹⁶.

amounts in three experiments. Only single residues were recovered at the other steps. Since position 10 corresponds to position 216 as indicated previously⁵, protein CRA has an internal deletion of about 200 residues. A tryptic amino-terminal glycopeptide was also sequenced (peptide T4 in ref. 5). Here, too, equal amounts of Leu and Ile were detected in position 3. It is likely that the carbohydrate moiety is attached to Asn at position 5. Accurate quantitation was based on the use of valine as an internal standard.

More than 90 heavy chain aminoterminal regions have been sequenced to date and on the basis of these studies, four V_H subclasses have been defined and all but one intact myeloma has been shown to fit into one of these⁸. In contrast, of the seven heavy chain disease proteins with internal deletions whose aminoterminal sequence is known^{5,9-11}, only three correspond exactly to one of the V_H subclasses⁸, while three have unusual substitutions or strikingly abnormal sequences. The three proteins described here seem to fall into two groups. Comparison of their sequence with the normal V_H sequence suggests that two (HAR and LEA) begin with residue 3. It is now accepted that L and probably H chains are synthesised as precursor molecules on membrane bound polyribosomes¹². The precursor contains a hydrophobic peptide of about 20 residues which is split when the chains are released¹³. The splitting seems to be specific, as the majority of secreted H chains have either pyrrolidone-carboxylic acid (PCA) as an aminoterminal residue or glutamic acid. In the case of protein HAR and LEA, it is conceivable that either their precursor is not entirely normal or that as a consequence of the deletion the molecule is folded differently thus allowing splitting to occur at the next available X-Gln peptide bond. This is supported by the finding of a peptide in protein LEA whose amino acid composition fits very well between positions 3 and 8 (Fig. 1). This short sequence is a common structure for V_HI and V_HIII subgroups⁸. The amino-

Fig. 1 The aminoterminal sequence of immunoglobulin human variant proteins with unusual aminoterminal sequences compared to V_HI and V_HIII subgroups. Amino acid sequence of protein CRA was performed by the method of Edman and Begg¹⁷ with a Beckman 890C sequencer using the Beckman Me₂ allylamine peptide programme (10/29/74). Edman degradation of CRA, aminoterminal tryptic peptide was done as described previously using dansylation to mark the new amino terminal residues^{15,16}. Residue 10 corresponds to position 216, γ l numbering¹⁸. CHO, carbohydrate.

	1	5	
V _H I	PCA-Val-Gln-Leu-Val-Glu-Ser-Gly		
V _H III	Glu-Val-Gln-Leu-Val-Glu-Ser-Gly		
LEA	PCA-Leu(Val, Glu, Ser, Gly)		
IIAR	PCA-Leu		
CRA	1	CHO	10
	Gly-Phe-Leu-Asp-Asn-Arg-Thr-Thr-Glu-Glu...		
		Ile	

terminal sequence of protein CRA cannot be placed in any known V region nor does it bear any obvious relation to any of the propeptides recently reported. The presence of equal amounts of Leu and Ile in position 3 suggests that two amino-terminal genes are expressed and joined to the same C gene as suggested previously for another H-chain variant¹¹. Whether this is due to the existence of two germ line V genes or a somatic mutation during the course of the disease is not known.

We thank Dr P. Majerus for protein LEA, Dr S.E. Maddison for protein HAR. This work was supported in part by US PHS grants AM 01431, AM 02594, and GM 20069.

B. FRANGIONE
E. C. FRANKLIN

Irvington House Institute,
Departments of Medicine and Pathology,
New York University Medical Center,
New York, New York 10016

O. SMITHIES

Department of Genetics,
University of Wisconsin,
Madison, Wisconsin 53706

Received 17 January; accepted 28 March 1978.

- Franklin, E. C., Lowenstein, J., Bigelow, B. & Meltzer, M. *Am. J. Med.* **4**, 332-350 (1964).
- Osserman, E. F. & Takatsuki, K. *Am. J. Med.* **37**, 351-373 (1964).
- Frangione, B. & Franklin, E. C. *Seminars Hematol.* **10**, 53-64 (1973).
- Adetugbo, K., Milstein, G. & Secher, D. S. *Nature* **265**, 299-304 (1977).
- Franklin, E. C. & Frangione, B. *Proc. natn. Acad. Sci. U.S.A.* **68**, 187-191 (1971).
- Lyons, R. M., Chaplin, H., Tillack, T. W. & Majerus, P. W. *Blood* **46**, 1-9 (1975).
- Press, E. M., Piggot, P. J. & Porter, R. R. *Biochem. J.* **99**, 356-366 (1966).
- Kabat, E. A., Wu, T. T. & Bilofsky, H. *Natn. Inst. Hlth. Division of Research Resources*, 1976.
- Franklin, E. C. & Frangione, B. in *Contemporary Topics in Molecular Immunology* (eds Inman, F. P. & Mandy, W.) 80-126 (Plenum, New York, 1975).
- Terry, W. D. & Ohms, J. *Proc. natn. Acad. Sci. U.S.A.* **66**, 558-563 (1970).
- Frangione, B. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1552-1555 (1976).
- Milstein, C., Brownlee, G. G., Harrison, T. M. & Mathews, M. B. *Nature new Biol.* **239**, 117-120 (1972).
- Burstein, Y. & Schechter, I. *Proc. natn. Acad. Sci. U.S.A.* **74**, 716-720 (1977).
- Weber, K. & Osborn, M. *J. biol. Chem.* **244**, 4406-4412 (1969).
- Gray, W. R. *Meth. Enzym.* **46**, 469-475 (1967).
- Woods, K. R. & Wang, K. T. *Biochim. biophys. Acta* **133**, 369-370 (1967).
- Edman, P. & Begg, G. *Eur. J. Biochem.* **1**, 80-91 (1967).
- Edelman, G. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **63**, 78 (1969).

Translation of *Xenopus* vitellogenin mRNA during primary and secondary induction

WHEN oestradiol is administered for the first time to male *Xenopus* the primary response is characterised by a latent period of 12-24 h before vitellogenin synthesis by the liver, or its secretion into the blood, is detected^{1,2}. The response reaches a maximum at 10-40 d then slowly declines until by 35-40 d the liver has ceased to produce the protein. A second injection at this stage (induction of secondary response) causes a rapid re-acquisition of vitellogenin synthesis with a much reduced lag period (3-12 h), with maximum levels of vitellogenin synthesis being reached much earlier^{1,3,4}. No satisfactory explanation has yet been found for this difference in lag periods, especially as RNA synthesis is known to be stimulated with little delay (2 h), even during primary induction^{5,6}. We therefore asked the question of whether the drastic reduction in the lag period could be due to differential rates of utilisation of vitellogenin mRNA induced by the hormones.

Induction of vitellogenin was monitored by measuring the appearance of ³⁵S-labelled proteins in male *Xenopus* plasma by SDS-polyacrylamide gel electrophoresis as well as by radioimmunoassay. As shown in Fig. 1, primary oestradiol injection caused a progressive increase over the first 6-10 d in the accumulation of labelled vitellogenin, which became the principal radioactive plasma protein by the third day after hormone administration. The failure to detect vitellogenin in the blood of uninduced animals (immunologically or by electrophoresis) confirms reports of the absence of secretion of this protein by the liver of control male *Xenopus*,

either *in vivo* or in cultures^{3,7,8}, and confirms recent studies showing none or almost no vitellogenin messenger RNA sequences using complementary DNA as a probe^{9,10}. At 40 d after the hormone, no labelled vitellogenin could be detected in the circulation. Figure 1 also shows that during the secondary response, newly synthesised vitellogenin was secreted into the plasma with shorter lag period (<12 h) than during primary stimulation and that it rapidly reached a peak of response by the third day after hormone administration, while maintaining the same rate of synthesis up to 12 d. Immunological analysis of the labelled plasma proteins also showed that the absolute incorporation of labelled amino acid into plasma albumin remained fairly constant during primary induction, but represented a much smaller fraction of total incorporation (Fig. 1). During secondary induction, however, the absolute rate of albumin synthesis declined quite significantly (Fig. 1b) but the reason for this

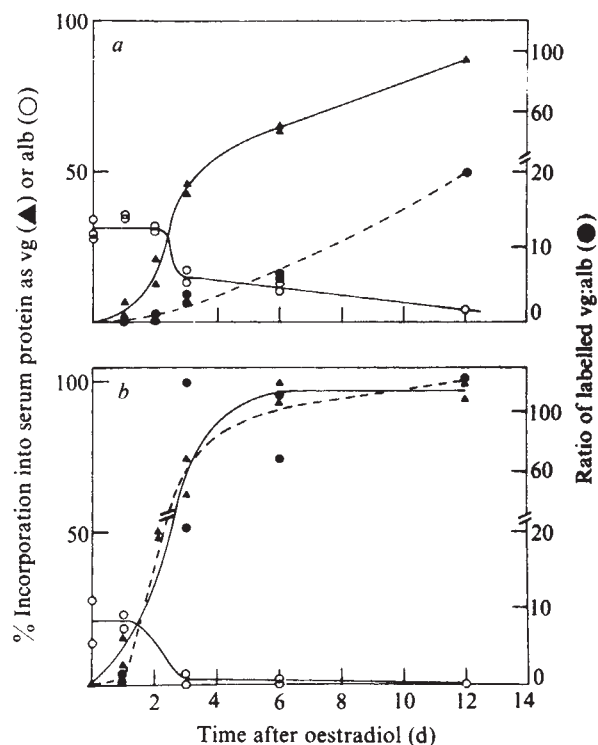


Fig. 1 Immunological quantitation of ³⁵S-labelled vitellogenin and albumin in male *Xenopus* plasma as a function of time during the onset of *a*, primary and *b*, secondary responses to 1 mg oestradiol-17 β (a second injection of 1 mg oestradiol was given to frogs treated with the same dose 41 d earlier). For each time point, groups of three male frogs were injected with 200 μ Ci of ³⁵S-methionine 18 h before withdrawing blood by cardiac puncture. Blood samples were diluted with an equal volume of citrated saline and the plasma derived from it with 4 volumes of phosphate-buffered saline (PBS). The ³⁵S-labelled vitellogenin and albumin were immunoprecipitated from 25 μ l (secondary response) or 50 μ l (primary response), diluted with 400 μ l of PBS in the presence of 1% Nonidet P-40 and 20 mM methionine, by adding 200 μ l of the corresponding antibody and 30 μ g of carrier vitellogenin and albumin and incubating at 0 $^{\circ}$ C for 16 h (ref. 4). Nonspecific immunoprecipitation was measured by incubating 200 μ l of goat anti-rabbit IgC antiserum and 200 μ g of carrier nonspecific rabbit IgC. Immunoprecipitates were washed three times with PBS containing 1% Nonidet P-40 and 20 mM methionine, dissolved in Soluene 350 solubiliser and the radioactivity measured. ³⁵S-labelled vitellogenin and albumin in plasma were also characterised by electrophoresis on SDS-7.5% polyacrylamide gels as described previously⁴. An aliquot of plasma equivalent to the amount taken for immunoprecipitation was also used for measuring the radioactivity incorporated into total protein. The radioactivity incorporated into total protein on day 0 was 83.5×10^3 and 67.0×10^3 c.p.m. per 50 μ l of plasma for primary and secondary responses, respectively. ○, Albumin (alb); ▲, vitellogenin (vg); ●, ratio of labelled vitellogenin to albumin.

'turning off' effect is not known. The latent period and the rate of synthesis of vitellogenin after induction with oestradiol, as described above, are compatible with results obtained earlier^{3,7,8}.

At less than 40–50 μg RNA per assay, the amount of vitellogenin synthesised was found to be proportional to the amount of hormone-induced *Xenopus* liver cytoplasmic RNA added to a rabbit reticulocyte lysate system. Thus, this system was used to determine the amount of vitellogenin mRNA in a particular RNA sample. Polyacrylamide gel electrophoresis followed by autoradiography of immunoprecipitates of translation products reacting with antibodies to vitellogenin⁴ showed that, whereas the RNA from induced animals gave a strong band for the 210,000 molecular weight subunit of vitellogenin, that from untreated animals failed to do so. Oestrogen administration was therefore found to cause the accumulation of substantial quantities of translatable mRNA for vitellogenin in the cytoplasm. When the products of translation in the rabbit reticulocyte cell-free assay were treated with antibodies

against albumin, in the same way as above, there was no significant difference in the amount of labelled albumin programmed by liver RNA from control and hormone treated *Xenopus* liver (results not shown).

An important requirement for studying preferential translation of a given mRNA is the ability to identify polyribosomes engaged in its translation. In conditions established in our laboratory⁴ for immunoprecipitation with monospecific antibodies of *Xenopus* liver polysomes engaged in the synthesis of vitellogenin and albumin, we have shown the existence of large polysomes, engaged in the synthesis of vitellogenin, in post-nuclear supernatants from hormone-induced animals. These were absent in untreated animals and, at the same time, there was little difference between control and hormone-treated animals in the fraction of polysomes engaged in the synthesis of albumin. Knowing that there was no detectable stored translatable mRNA for vitellogenin in the cytoplasm of untreated control *Xenopus* liver, we asked two questions. (1) Is the lag period of vitellogenin synthesis during primary induction *in vivo* due to the inability of the cell to translate the newly-transcribed vitellogenin mRNA? And (2), can the absence of a similar lag period during secondary induction be explained by the persistence of untranslated mRNA formed during primary induction or by the translational impairment being somehow 'permanently' overcome? The following experiments resolve both these questions.

RNA extracted from the post-nuclear fraction of livers of male frogs at various times during primary and secondary responses to oestrogen was assayed for its vitellogenin and albumin mRNA contents. In a parallel experiment, total RNA in polysomes was labelled *in vivo* with a 24-h pulse of ³H-orotic acid and the fraction of polysomes engaged in the synthesis of vitellogenin and albumin was determined by specific immunoprecipitation. As shown in Fig. 2, the rate of appearance of vitellogenin mRNA during primary induction was rapid, reaching 60%, 80% and 90% of the maximum values at 48, 72 and 120 h after hormone administration, respectively. More important, this rapid build-up of mRNA levels in the cytoplasm followed similar kinetics at the onset of both primary and secondary responses (compare Fig. 2a and b), although it is quite likely that there may be some differences in the rate of accumulation of mRNA at the very early times, say within the first 12 h after hormone administration. Using a complementary DNA probe to quantitate the accumulation of mRNA, two recent studies have indeed reported differences in the rate of accumulation of vitellogenin message sequences during the first 12 h after primary and secondary responses in *Xenopus*¹¹ and chicken¹² liver. However, the gross similarity in the pattern of the onset of primary and secondary responses, in terms of translatable cytoplasmic vitellogenin message, did not hold when we determined by immunoprecipitation the fraction of vitellogenin mRNA in labelled polysomes. During primary response, the fraction of polysomes engaged in vitellogenin synthesis rose only slowly reaching 25% of the maximum value at day 3 and 100% at 12 d after hormone administration, which contrasts with the findings of Shapiro *et al.*¹³ that there was no lag between vitellogenin mRNA levels and its translation. At 35 d after the first administration of oestrogen, both the levels of vitellogenin mRNA and fraction of polysomes engaged in its translation fell to 0–10% of the maximum levels. In contrast to primary induction, the recruitment of message into active polysomes formed during secondary response was a function of the rate of appearance of vitellogenin mRNA.

When the levels of mRNA for albumin and the fraction of total polysomes engaged in its synthesis were determined at various times during primary and secondary response to oestradiol, changes similar to those mentioned above for vitellogenin were not observed (Fig. 3). There was, however, a noticeable drop in albumin mRNA and polysomes 6 d

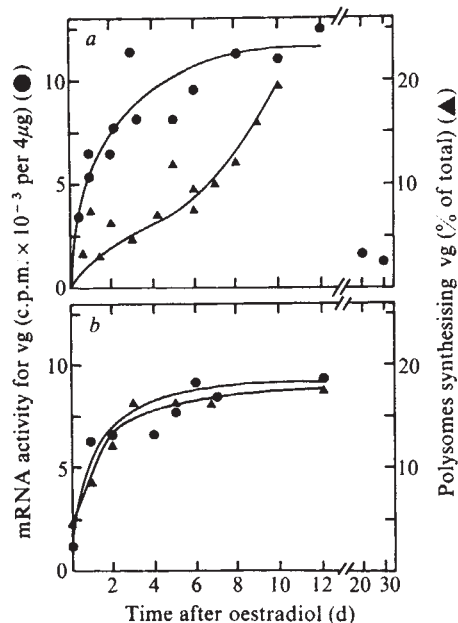


Fig. 2 Simultaneous measurement of changes in levels of vitellogenin (vg) mRNA and of polysomes engaged in its translation in liver of male *Xenopus* at different stages during a, primary and b, secondary (35 d after the first injection of oestradiol) responses to oestradiol *in vivo*. It was first established that the rabbit reticulocyte assay system used in these experiments exhibited a linear response to added *Xenopus* vitellogenin or albumin mRNA up to 40 μg of total RNA. 40 μg total post-nuclear RNA obtained at different stages of primary and secondary responses were therefore assayed in a reticulocyte cell-free system for vitellogenin mRNA activity by immunoprecipitation of ³⁵S-methionine-labelled translation product, with anti-vitellogenin antibody. Polysomes were labelled with a 24-h pulse of 200 μCi of ³H-orotic acid per frog and the fraction engaged in synthesis of vitellogenin was specifically immunoprecipitated at 4 °C by incubating up to 4 A_{260} units of polysomes in PBS containing 1% Nonidet P-40 with increasing concentrations of anti-vitellogenin or nonspecific rabbit γ -globulins. A saturation curve for each polysome preparation was determined in order to ensure maximum precipitation of polysomes. 250–1,000 μg of γ -globulin would quantitatively precipitate polysomes from a total input of 3 A_{260} units. Excess goat anti-rabbit serum (500 μl per 500 μg of rabbit γ -globulin) was added after an initial incubation of 60 min. The mixture was left for 18 h and then centrifuged at 3,000 g for 10 min. The immunoprecipitates were washed twice with ice-cold PBS containing 1% Nonidet P-40, dissolved in 0.5 ml of NCS tissue solubiliser and the radioactivity measured in a scintillation counter. ●, Level of translatable vitellogenin mRNA; ▲, fraction of total polysomes engaged in synthesising vitellogenin.

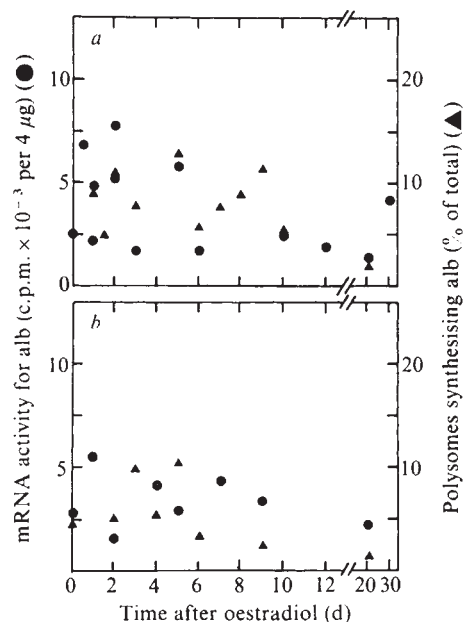


Fig. 3 Changes in levels of albumin (alb) mRNA and of polysomes engaged in its translation at different stages of *a*, primary and *b*, secondary responses to oestradiol. The same samples of liver as those used in the experiments shown in Fig. 2 were used, except that anti-albumin antibodies replaced those directed against vitellogenin and that the optimum conditions were first established for the maximum precipitation of polysomes engaged in the synthesis of albumin. All experimental conditions were the same as those in Fig. 2. ●, Albumin mRNA; ▲, albumin synthesising polysomes.

after the first or second oestradiol injection but the wide variations of values do not allow us to draw a firm conclusion. Nevertheless, the striking difference in kinetics of synthesis of the two proteins shown in Figs 2 and 3 support our conclusion that some post-transcriptional regulation is involved in vitellogenin production during primary stimulation and that the latent period preceding vitellogenin synthesis during primary induction by oestradiol may be due to the build-up of components required for the translation of the induced messenger RNA.

The current consensus of opinion regarding the induction of specific protein synthesis by steroid hormones is that hormonal regulation can largely be explained by transcriptional control, that is, that the rate of induced protein synthesis is a function of the cytoplasmic concentration of the mRNA, although some minor modulation of translation of inactive mRNA cannot be ruled out¹⁴. Our results agree with this conclusion as far as secondary induction of *Xenopus* vitellogenin is concerned, but not for the primary induction of vitellogenin synthesis. Our studies further suggest that during primary induction, a change in the level or nature of some translational component(s) would be relatively permanently 'imprinted' so that subsequent secondary stimulation only involves replenishment of the vitellogenin mRNA, which has an estimated half life in *Xenopus* liver of 40 h (refs 1, 3). The facilitation of translation of vitellogenin mRNA seems to be quite selective, as the rate of synthesis of albumin is either unaffected or diminished. What component(s) of the protein synthetic machinery is involved in establishing the preferential translation of vitellogenin mRNA during primary induction is not clear. Previous studies¹⁵ indicated the requirement of some 'soluble' cell sap factor(s) for the optimal activity *in vitro* of polysomes from oestrogen-treated male *Xenopus* liver. Studies on avian vitellogenin have also indicated that multiple seryl tRNAs may play a special part in the induction

of egg-yolk protein by oestrogen¹⁶. A totally different, but not mutually exclusive, explanation may lie in the relatively slow build-up of ribosomes^{4,17} and of the membranes of the endoplasmic reticulum¹⁷ on which vitellogenin is synthesised, chemically modified and prepared for secretion^{1,2}. Ultrastructural studies during primary induction have shown that the coordinated proliferation of membranes of the rough endoplasmic reticulum and membrane-bound ribosomes is slower than the appearance of mRNA for vitellogenin during primary induction¹⁷ with kinetics paralleling those of the synthesis and secretion of vitellogenin into the blood. Whatever the reason for the lag in translation of mRNA for vitellogenin, the results presented here show that transcriptional, as well as post-transcriptional or translational mechanisms underlie the kinetics of the expression of the vitellogenin gene during primary induction.

We thank Mr R. Lofthouse for technical assistance. S.R.F. was an MRC scholar.

S. R. FARMER*
E. C. HENSHAW†
M. V. BERRIDGE‡
J. R. TATA

National Institute for Medical Research,
Mill Hill, London NW7, UK

Present addresses:

*Department of Biology, M.I.T., Cambridge, Massachusetts 02139.

†Cancer Center of University of Rochester, Rochester, New York 14642.

‡Department of Biochemistry, University of Wellington, Wellington, New Zealand.

Received 23 December 1977; accepted 10 April 1978.

1. Clemens, M. J. *Prog. Biophys. molec. Biol.* **28**, 71-107 (1974).
2. Tata, J. R. *Cell* **9**, 1-14 (1976).
3. Clemens, M. J., Lofthouse, R. & Tata, J. R. *J. biol. Chem.* **250**, 2213-2218 (1975).
4. Berridge, M. V., Farmer, S. R., Green, C. D., Henshaw, E. C. & Tata, J. R. *Eur. J. Biochem.* **62**, 161-171 (1976).
5. Whittliff, J. L., Kai-Lin, L. & Kenney, F. T. *Biochim. biophys. Acta* **269**, 493-504 (1972).
6. Tata, J. R. & Baker, B. *Biochem. J.* **150**, 345-355 (1975).
7. Green, C. D. & Tata, J. R. *Cell* **7**, 131-139 (1976).
8. Whittliff, J. L. & Kenney, F. T. *Biochim. biophys. Acta* **269**, 485-492 (1972).
9. Ryffel, G., Wahli, W. & Weber, R. *Cell* **11**, 213-221 (1977).
10. Shapiro, D. J. & Baker, H. J. *J. biol. Chem.* **252**, 5241-5250 (1977).
11. Baker, H. J. & Shapiro, D. J. *J. biol. Chem.* **252**, 8428-8433 (1977).
12. Deeley, R. G., Udell, D. S., Burns, A. T. H., Gordon, J. I. & Goldberger, R. F. *J. biol. Chem.* **252**, 7913-7915 (1977).
13. Shapiro, D. J., Baker, H. J. & Stitt, D. T. *J. biol. Chem.* **251**, 3105-3111 (1976).
14. Palmiter, R. D. *Cell* **4**, 189-197 (1975).
15. Clemens, M. J. & Tata, J. R. *Eur. J. Biochem.* **33**, 71-80 (1973).
16. Maenpaa, P. H. & Bernfield, M. R. *Biochemistry* **8**, 4926-4935 (1969).
17. Lewis, J. A., Clemens, M. J. & Tata, J. R. *Molec. Cell. Endocr.* **4**, 311-329 (1976).

Preferential binding of hog brain microtubule-associated proteins to mouse satellite versus bulk DNA preparations

MICROTUBULES, as major constituents of the mitotic spindle, have been implicated in chromosome movement. It is not known, however, whether spindle microtubules interact directly with chromosome components. An electron microscopically distinct structure, the kinetochore, has been characterised as the chromosome-associated focus of microtubules that connect the chromosome to the spindle pole (see ref. 1 for review). However, very little is known about the nature of the kinetochore constituents and the possible interaction mechanisms². We show here that microtubule-associated proteins bind preferentially to satellite DNA sequences in the eukaryotic chromosome.

In an attempt^{3,4} to identify the interacting components of the spindle apparatus and the chromosome we found that microtubule proteins strongly bind to purified DNA. Binding studies after fractionation of microtubule proteins into tubulin and microtubule-associated proteins (MAPs) revealed that tubulin alone is not capable of binding to DNA, but tubulin does bind to DNA in the presence of MAPs. As

purified MAPs themselves also exhibited a strong binding capacity we proposed that the binding of microtubules to chromosomes might take place through the binding of MAPs to DNA³. This hypothesis is further supported by the results of Conolly *et al.*⁵ which indicated that MAPs are associated with microtubules not only *in vitro*⁶⁻⁸ but also *in vivo*.

The questions arise as to whether MAPs bind to DNA in a random fashion, that is, all along the DNA strand, or whether they preferentially bind to certain base sequences or domains of DNA. For several reasons stretches of satellite DNA seem likely candidates for preferential binding sites. First, they are distinguished from the bulk of the DNA by consisting of short base sequences, strung together in long tandem repeats⁹, thus providing potential binding sites for proteins not involved in gene transcription. Furthermore, in the mouse and guinea pig they are found in the condensed fraction of isolated interphase chromatin^{10,11} and in mouse in the less soluble fraction of metaphase chromosomes¹² and near¹³ the centromere (kinetochore). Finally, in line with these findings we showed recently¹ that MAPs preferentially bound to DNA that contained satellite DNA sequences (eukaryotic) as compared to DNA that lacked such sequences (prokaryotic).

Figure 1 shows the fractionation of ³H-thymidine labelled total mouse 3T6 fibroblast DNA into satellite and bulk DNA fractions in Ag⁺-Cs₂SO₄ density gradients. The peak fractions were pooled as indicated, desalted by dialysis against 0.1×SSC (ref. 14) and samples of the DNA then used in binding assays. In control experiments sucrose gradient centrifugation showed that the size distributions of DNA fragments in satellite and bulk fractions were very similar (data not shown). MAP preparations were obtained by fractionation of microtubule proteins purified from hog brain extracts by repeated cycles of *in vitro* polymerisation according to the procedure of Shelanski *et al.*¹⁹. The separation of MAPs from tubulin was achieved in two steps. First, the bulk of tubulin was separated by phosphocellulose column chromatography as described by Weingarten *et al.*⁹. The MAP-containing eluates from the columns were then dialysed against a solution containing 100 mM sodium *N*-morpholinoethanesulphonate (MES), 0.1 mM MgCl₂ and 0.1 mM GTP and rechromatographed on DEAE-Sephadex columns⁷, that had been equilibrated with the same buffer; MAPs were eluted with buffer containing 0.3 M potassium chloride. As judged by SDS-polyacrylamide gel electrophoresis, these MAP fractions contained several proteins, including relatively high amounts of two of quite high molecular weight⁷, but no tubulin. Several such protein preparations were used in this study; all exhibited the capacity to bind to DNA, although the efficiency of their binding varied somewhat and was found to depend on factors such as the age of the preparation tested. MAP preparations that had been purified by only one step of column chromatography (phosphocellulose) in binding studies behaved in a similar manner to those purified by chromatography in two steps (phosphocellulose plus DEAE-Sephadex).

To find whether the protein preparations showed a preference in binding to any of the DNA fractions prepared in Ag⁺-Cs₂SO₄ density gradients, increasing amounts (protein was determined according to the method of Lowry *et al.*²⁰) of a MAP preparation were incubated with constant amounts of ³H-labelled satellite or bulk DNA samples, and the complexes formed were isolated on nitrocellulose filters²¹. If MAPs preferentially bound to satellite versus bulk DNA, then equal amounts of protein would bind relatively more satellite than bulk DNA: this was found to be the case (Fig. 2). MAPs bound about twice as effectively to satellite as to bulk DNA preparations.

To substantiate this result the binding of MAPs to fractions of DNA was also tested in competitive fashion. Satellite and bulk DNA fractions were prepared from un-

labelled total 3T6 cell DNA and samples were incubated with constant amounts of MAPs and ³H-labelled satellite DNA and the extent of binding was again determined by filter binding assays. The amount of MAPs added was far below that required for binding of all the DNA material in the assay mixtures, so relatively less labelled satellite DNA should be found bound to MAPs (and hence to the nitrocellulose filters) if one of the added unlabelled DNA samples was competing more effectively for binding than the other. As shown in Fig. 3, unlabelled satellite DNA competed with labelled satellite DNA to a significantly greater extent than did bulk DNA. Control experiments, run in parallel, showed that the ability of phage ϕ 29 DNA to compete with satellite DNA for binding was even lower than that of bulk DNA. In the competition experiments an average of slightly less

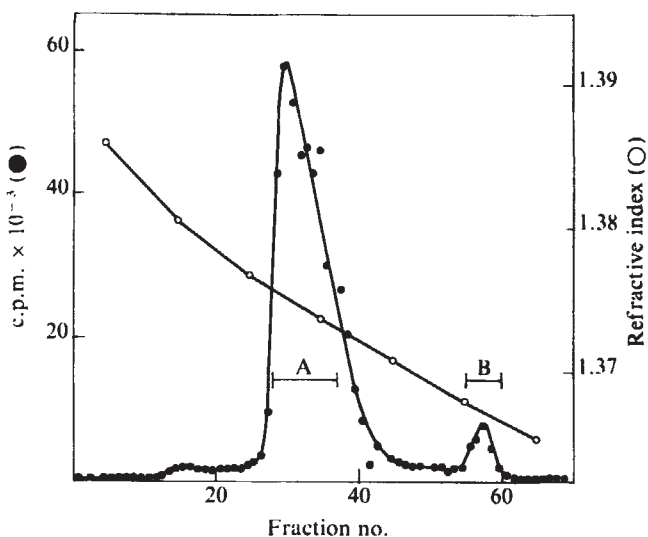


Fig. 1 Fractionation of total mouse 3T6 cell DNA on Ag⁺-Cs₂SO₄ density gradient. Mouse 3T6 fibroblast cells (from H. Green) were grown in roller bottles in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. For the preparation of labelled cellular DNA cells were seeded at relatively low densities and the original growth medium was replaced by fresh medium containing 5 μ Ci ml⁻¹ ³H-thymidine (Radiochemical Centre) after 24 h of growth; cells were then grown to confluence (3 d). Cells were washed twice with 10 mM sodium phosphate, pH 7.2, 0.15 M sodium chloride and lysed by the addition of 15 ml (per bottle) 10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 10 mM sodium chloride; and 0.5% sodium dodecyl sulphate¹³. The cell lysates were incubated with 50 μ g ml⁻¹ proteinase K (Merck, Darmstadt) overnight at 37 °C. Total DNA was prepared by a method slightly modified from that of Marmur¹⁶; DNA was extracted three times with a mixture of chloroform-isoamyl alcohol (12:1) and finally precipitated by 2 volumes of ethanol. The precipitate was air dried, dissolved in 0.1×SSC (ref. 14) and treated in sequence with 50 μ g ml⁻¹ RNase (pretreated as described in ref. 18) for 1.5 h and 50 μ g ml⁻¹ proteinase K for 3 h. DNA was then again extracted with chloroform isoamyl alcohol, precipitated with ethanol, dried and finally dissolved in 5 mM borate buffer, pH 9.2. Total ³H-labelled cell DNA (45 μ Ci mg⁻¹) was fractionated by the procedure of Corneo *et al.*¹⁷ as modified by Botchan¹⁸. The mixtures to be centrifuged contained DNA at an A₂₆₀ of 0.6, 4.25 μ g ml⁻¹ AgNO₃, 5 mM borate, pH 9.2 and Cs₂SO₄ (Merck, Darmstadt) to give a final density of about 1.5 g ml⁻¹. Samples of 9 ml were spun in a Beckman 40 rotor at 35,000 r.p.m. for 90 h. Fractions (6 drops) of the gradients were then collected and aliquots used for counting radioactivities or measuring refractive indices. Unlabelled total 3T6 DNA was prepared and fractionated in a similar manner except that the cells were grown in the absence of ³H-thymidine and small amounts of ³²P-labelled total 3T6 cell DNA were mixed to the unlabelled DNA before fractionation for tracing. A, bulk DNA; B, satellite DNA.

than one seventh of labelled satellite DNA was found bound to filters in the presence of a 6.8-fold excess of unlabelled satellite DNA; this corresponds well to the expected value. These results were confirmed by similar competition experiments with ^3H -labelled satellite DNA and unlabelled, unfractionated DNA samples that either contained (such as hog liver) or lacked (phage) satellite sequences (ref. 3 and G.W., V.G.C., J.A., unpublished).

Our finding that hog brain MAPs *in vitro* preferentially associate with satellite DNA suggests that satellite DNA sequences might be the attachment site for microtubules *in vivo* and that MAPs are the linking bridges. In these studies we used heterologous MAP preparations (hog brain) as such preparations are relatively stable and the proteins can therefore be isolated in functional states^{6,7}. In contrast, studies with a cultured cell line (rat glial C6) indicated that MAPs from cultured cells are extremely unstable and that it is impossible to isolate them in functional forms from such a source by following established⁶ procedures (ref. 22 and G.W., L. S. Honig and R. D. Cole, unpublished).

Although our experiments clearly showed that the binding of MAPs is preferential to satellite versus bulk DNA fractions, the observed difference was only about twofold. However, this difference could be sufficient for site-dependent microtubule formation at the kinetochore. Several studies of *in vitro* self-assembly of microtubules have revealed that a 'critical' concentration of tubulin must be exceeded in order to initiate microtubule assembly. Cleveland *et al.*²³ have recently demonstrated that MAPs bring down this critical concentration in a concentration-dependent manner. Thus any accumulation of MAPs at binding sites on satellite DNA could have similar effects on spindle-microtubule formation.

Fig. 2 Binding of MAPs to satellite and bulk DNA fractions. Equal aliquots (0.05 μg) of tritiated satellite or bulk DNA and the indicated amounts of a MAP preparation that had been purified by two-step column chromatography were mixed in a total volume of 100 μl of binding buffer (25 mM sodium *N*-morpholinoethanesulphate pH 7.0, 75 mM KCl, 0.025 mM MgCl_2 , 0.8 mM NaCl, 0.08 mM sodium citrate) and incubated at 35 $^\circ\text{C}$ for 10 min. The mixtures were then applied to nitrocellulose filters (Millipore HAWP, 0.45 μm) that had been equilibrated in binding buffer plus 1% dimethyl sulphoxide to assay for binding. Sample tubes and filters were rinsed with a total of 6 ml of binding buffer, the filters dried, immersed in scintillation fluid and counted. ●, Satellite DNA; ○, bulk DNA.

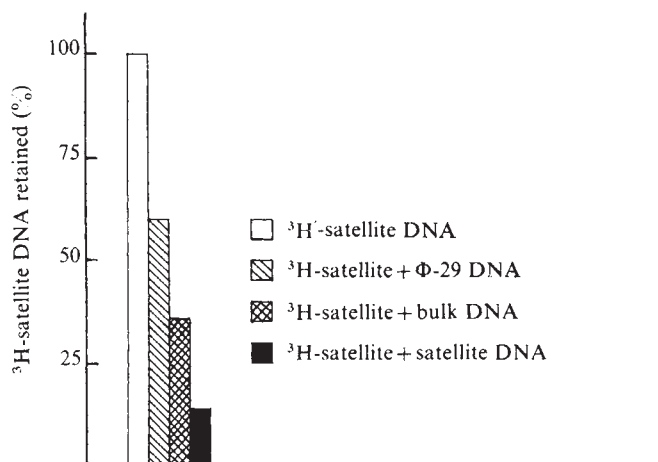
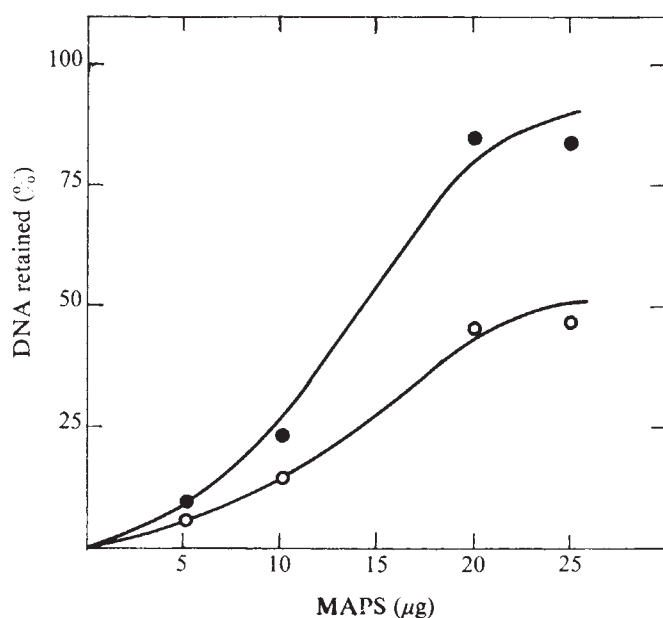


Fig. 3 Competitive binding of satellite and bulk DNA fractions to MAPs. ^3H -satellite DNA fractions were mixed with unlabelled preparations of either satellite, bulk or total phage Φ 29 DNA. In all cases the ratios (w/w) of labelled to unlabelled DNA were 1 : 6.8. MAPs, prepared as described in Fig. 2, were then added to these mixtures to yield ^3H -satellite DNA protein ratios (w/w) of 1 : 200. The conditions of incubation and subsequent filter binding assays were as described in Fig. 2 except that the total volumes of the incubation mixtures varied in the different experiments. Each bar represents the average of three independent experiments. The amount of ^3H -satellite DNA bound to filters in the absence of unlabelled DNA was taken as 100% binding (bar). Binding levels and standard deviations were $14 \pm 8\%$ (solid bar), $36 \pm 10\%$ (cross hatched bar) and $60 \pm 20\%$ (single lined bar).

We thank Dr E. Vinuela for helpful suggestions and Dr M. Salas for a gift of phage Φ 29 DNA. This work was supported by grants from the Comisión Asesora para el Desarrollo de la Investigación Científica y Técnica and Comisión Administradora del Descuento Complementario (I.N.P.). G.W. was supported by an EMBO short-term fellowship.

GERHARD WICHE*
VICTOR G. CORCES
JESUS AVILA

Centro de Biología Molecular, CSIC,
Universidad Autónoma de Madrid,
Madrid-34, Spain

Received 9 December 1977; accepted 22 March 1978.

*To whom correspondence should be addressed. Present address: Institute of Biochemistry, University of Vienna, Währingerstr. 17, 1090 Vienna, Austria.

- Brinkley, B. R. & Stubblefield, E. *Adv. Cell Biol.* **1**, 119-185 (1969).
- Nicklas, B. R. *Adv. Cell Biol.* **2**, 225-297 (1971).
- Corces, V. G., Salas, J., Salas, M. L. & Avila, J. *Eur. J. Biochem.* (in the press).
- Corces, V. G. & Avila, J. *Eur. J. Biochem.* (in the press).
- Conolly, J. A., Kalnins, V. I., Cleveland, D. W. & Kirschner, M. W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2437-2440 (1977).
- Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. & Kirschner, M. W. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1858-1862 (1975).
- Murphy, D. B. & Borisy, G. G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2696-2700 (1975).
- Keates, R. A. B. & Hall, H. R. *Nature* **257**, 418-421 (1975).
- Southern, E. M. *Nature* **227**, 794-798 (1970).
- Yasminieh, W. G. & Yunis, J. J. *J. Exp. Cell Res.* **59**, 69-75 (1970).
- Yunis, J. J. & Yasminieh, W. G. *Science* **168**, 263-265 (1970).
- Maio, J. J. & Schildkraut, C. L. *J. molec. Biol.* **40**, 203-216 (1969).
- Jones, K. W. *Nature* **225**, 912-915 (1970).
- Gillespie, D. & Spiegelman, S. *J. molec. Biol.* **12**, 829-842 (1965).
- Gross-Bellard, M., Oudet, P. & Chambon, P. *Eur. J. Biochem.* **36**, 32-38 (1973).
- Marmur, J. *J. molec. Biol.* **3**, 208-218 (1961).
- Corneo, G., Ginelli, E., Soave, C. & Bernardi, G. *Biochemistry* **7**, 4347-4379 (1968).
- Botchan, M., McKenna, G. & Sharp, P. *Cold Spring Harb. Symp. quant. Biol.* **38**, 383-395 (1974).
- Shelanski, M. L., Gaskin, F. & Cantor, C. R. *Proc. natn. Acad. Sci. U.S.A.* **70**, 765-768 (1973).
- Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265-275 (1951).
- Jones, D. N. & Berg, P. J. *J. molec. Biol.* **22**, 199-209 (1966).
- Wiche, G., Honig, L. S. & Cole, R. D. *Nature* **269**, 435-436 (1977).
- Cleveland, D. W., Hwo, S. Y. & Kirschner, M. W. *J. molec. Biol.* **116**, 207-225 (1977).

matters arising

Phase transitions and indentation hardness of Ge and diamond

SINCE publication of our letter¹ on phase transitions and indentation hardness of Ge, Si and diamond our attention has been drawn to the paper by Gridneva *et al.*² on the hardness of diamond-like solids. They directly confirm the formation of a metallic phase below the indenter due to the high hydrostatic pressure generated in the contact zone. Ruoff³ has expressed some doubts as to the occurrence of a metallic transition in the experiments of Vereshchagin *et al.*⁴. However, in discussion of a more recent paper he notes that with small spherical indenters indentation pressures as high as 19,000 kg mm⁻² might be reached⁵; this exceeds the value of 17,000 kg mm⁻² estimated by van Vechten⁶ as the pressure required to produce the metallic phase in diamond. The concept of a metallic phase below the indenter is also implied by Gilman⁷ who proposes that the resistance to flow is determined by the need for two bonding electrons to be promoted into the conduction band. This he relates to the band gap. We have used the band gap⁸ rather than the band gap as this provides a better measure of the average energy to break the covalent bond.

D. TABOR

Department of Physics,
University of Cambridge,
Madingley Road,
Cambridge, UK

1. Gerk, A. P. & Tabor, D. *Nature* **271**, 732 (1978).
2. Gridneva, I. V., Milman, Yu. V. & Trefilov, V. I. *Phys. stat. sol. A* **14**, 177 (1972).
3. Ruoff, A. L. *Rev. pap. 6th AIRAPT Int. High Pressure Conf.* Boulder, Colorado (1977).
4. Vereshchagin, L. F. *et al. High Temp.-High Press.* **6**, 499 (1974).
5. Ruoff, A. L. *Science* **198**, 1037 (1977).
6. van Vechten, J. A. *Phys. Rev. B* **7**, 1479 (1973).
7. Gilman, J. J. *J. appl. Phys.* **46**, 5110 (1975).
8. Phillips, J. C. *Bonds and Bands in Semiconductors* (Academic, New York, 1973).

Oxygen production from chlorophyll-liposomes

THE report of Toyoshima *et al.*¹ on the evolution of oxygen with chlorophyll-containing liposomes is of considerable interest. We have been unsuccessful in our attempts at doing essentially the same experiments². In contrast, we readily measured oxygen uptake by chlorophyll-liposomes as the pigments became irreversibly photooxidised³. In addition to ferricyanide (the Hill acceptor used by Toyoshima), we used

several other electron acceptors and could always measure oxygen consumption (Fig. 1), and as well as the basic experiment reported by Toyoshima, we tested many buffers, salts, pH's, temperatures, electron donors and acceptors and membrane components, and always observed uptake.

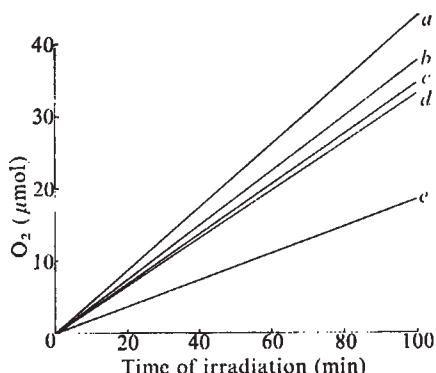


Fig. 1 The effect of electron acceptors on oxygen consumption with chlorophyll-liposomes. *a*, 1 mM dehydroascorbate; *b*, 1 mM methyl viologen; *c*, no addition (control); *d*, 1 mM ferricyanide; *e*, 1 mM benzoquinone.

Toyoshima *et al.* prepared their liposomes in 0.1 M potassium ferricyanide, 0.5 M Tris and 0.1 M KCl at pH 7.5, and separated the unsequestered ferricyanide from the ferricyanide-encapsulated liposomes on a Sephadex G-50 column presaturated with the washing buffer, 0.5 M Tris and 0.1 M KCl at pH 7.5. The problem is that the ionic strength of the internal ferricyanide is not compensated for in the washing buffer. It is hard to imagine the ferricyanide-encapsulated liposomes not swelling and breaking, and releasing the trapped ferricyanide on passage down the column. Additionally, 0.5 M Tris is a poor buffer for relevant oxygen evolution studies as large quantities of Tris are known to destroy oxygen evolution *in vivo*⁴.

Our main criticism centres around Toyoshima's total lack of temperature controls. We were not able to measure any oxygen evolution with the stringent temperature controls (usually 25 ± 0.1 °C) used in our experiments. However, we did carry out experiments in which we allowed the temperature of the chlorophyll-liposomes to rise in the dark. In these conditions, we were able to measure apparent 'oxygen' at levels similar to those reported in Fig. 2 of Toyoshima, with temperature increases of between 0.5 and about 4 °C. As Toyoshima employed a 500-W xenon

lamp, the possibility of heating artefacts remains quite real.

We believe the experiments reported by Toyoshima *et al.* should be repeated using stringent temperature controls and different buffers, and also checking the effect of classic oxygen inhibitors like DCMU and hydroxylamine, and oxygen stimulators like Mn²⁺ and bicarbonate⁵.

W. STILLWELL
H. TI TIEN

Biophysics Department,
Michigan State University,
East Lansing, Michigan 48824

1. Toyoshima, Y., Morino, M., Motoki, H. & Sukigara, M. *Nature* **265**, 187-189 (1977).
2. Tien, H. T. in *MTP International Review of Science* Vol. 6 (ed. Kerker, M.) 56 (Butterworths, London, 1972).
3. Stillwell, W. & Tien, H. T. *Biochim. biophys. Acta* **461**, 239-252 (1977).
4. Yamashita, T. & Butler, W. L. *Pl. Physiol.* **43**, 1978-1987 (1968).
5. Tien, H. T. in *Photosynthetic Oxygen Evolution* (ed. Metzner, H.) (Academic, New York, in the press).

Energy flow and the number of trophic levels in ecological communities

Pimm and Lawton¹ showed, using Monte Carlo simulation methods, that the return times to equilibrium, T_R , following small perturbations to ecological food chain populations modelled by Lotka-Volterra equations, are positively correlated with the lengths, L , of the food chains. We have made similar studies² showing T_R to be strongly positively correlated with $T_M(1-n)$, the mean transit time of a molecule or unit of energy from the bottom (autotroph) to the top (top carnivore) of the food chain; T_R is positively correlated with L only if $T_M(1-n)$ is positively correlated with L , which need not always be the case.

Our point can be made analytically for the simple food chain of n species,

$$\frac{dX_i}{dt} = X_i(b_i - \sum_{j=1}^n a_{ij}X_j) \quad (i = 1, 2, \dots, n)$$

where we assume $b_1 > 0$, $b_i = 0$ ($i > 1$), since only the bottom species is autotrophic. It can be shown³ that the equilibrium densities, X_i^* , are proportional to b_1 , and that the eigenvalues of the equations linearised about the equilibrium values are also proportional to b_1 . The return time, T_R , of the perturbed system is roughly proportional to $-1/\text{Real}(\lambda_{\max})$, where $\text{Real}(\lambda_{\max})$ is the largest real part of any of the eigenvalues of the system. The transit times of a molecule up the chain from one species

to the next obey the relations $T_M(i-j) \sim 1/X_j^* a_{ji} \sim 1/b_1$. Therefore, $T_R \sim T_M(1-n)$ for the Lotka-Volterra equations.

If longer food chains invariably imply a larger $T_M(1-n)$, then Pimm and Lawton's¹ results follow. It is feasible, however, that some long food chains could have smaller values of $T_M(1-n)$ than shorter food chains, if the former are associated with larger values of b_1 (have larger energy fluxes). Hence, long food chains could have shorter return times to equilibrium than short ones if energy is pumped through fast enough.

This research was sponsored by the Department of Energy under contract with Union Carbide Corporation.

D. L. DEANGELIS

R. H. GARDNER

Environmental Sciences Division

J. B. MANKIN

Computer Sciences Division

W. M. POST

Environmental Sciences Division

J. H. CARNEY

Computer Sciences Division

Oak Ridge National Laboratory

Oak Ridge, Tennessee 37830

1. Pimm, S. L. & Lawton, J. H. *Nature* **268**, 329-331 (1977).

2. Gardner, R. H., Mankin, J. B., DeAngelis, D. L., Post, W. M. & Carney, J. H. (in preparation).

Climatic trends in the North Atlantic

KUKLA *et al.*¹ in their article on climatic trends made no mention of the Panulirus series of bi-weekly observations taken off Bermuda (32° 10' N, 64° 30' W) since June 7 1954. Following the withdrawal of US weatherships in 1973, it is the only regularly reporting deep ocean (3,000 m) station in the North Atlantic north of the tropics and south of 50° N. Schroeder and Stommel² considered the question of how representative the series is of monthly mean conditions off Bermuda and concluded, on the basis of the first decade of data (1955-64), that the sampling frequency was dense enough to show real periodic phenomena such as anomalies of temperature. I have previously³ described the annual cycle of temperature, salinity, oxicity and density. It takes until February of the next year for summer-warmed surface (T_{max} at surface in August) water to mix down to 200 m, whereas winter-cooled (T_{min} in March) water penetrates more rapidly to 200 m in 3 months. Below 200 m it becomes difficult to see annual effects but they are discernible to 300 m for summer-warmed and 600 m for winter-cooled water. Salinity shows a seasonal cycle with a subsurface maximum which sinks from the surface in March

to 150 m by January. Having identified the main features of the annual cycle, we then looked for changes not within but between years⁴. Cubic spline interpolation was applied at standard depths to 800 m. The data were then regularised to monthly intervals. The resulting series does not accentuate excursions about the mean.

To determine whether there were significant long-term trends in the data, linear regression of parameters against time were recorded until the end of 1972. The slopes were tested for significant differences from a slope of zero that is, a line parallel to the ordinate signifying no consistent change in time.

Single months were tested for trend in temperature as was the complete record at each standard depth. All months showed a negative trend of monthly mean temperature against time (for example, at 1 m, September $-0.03^\circ\text{C yr}^{-1}$; May $-0.035^\circ\text{C yr}^{-1}$; April $-0.04^\circ\text{C yr}^{-1}$; June $-0.05^\circ\text{C yr}^{-1}$) except August and July. Whole year average decreased by $0.029^\circ\text{C yr}^{-1}$ at 1 m, $0.041^\circ\text{C yr}^{-1}$ at 100 m to $0.032^\circ\text{C yr}^{-1}$ at 350 m. The conclusion is inescapable: temperature of the water column off Bermuda has decreased over 18 yr by 2-3% of the mean. This trend is, incidentally, also shown by the unregularised data and is not an artefact of the treatment of the data. Salinity, with the exception of February, shows a consistently positive trend of high statistical significance in the top 150 m. Oxicity increased in all months except September.

These trends in three variables are related and do not need a separate explanation. The increase in dissolved oxygen is a predictable consequence of decreasing temperature and increasing salinity. Decreased insolation could explain lowered mean annual near-surface temperature but not increased salinity. Increase in wind stress could account for both lower annual temperature, due to increased mixing down of winter-cooled water and increasing salinity as a consequence of either increased evaporation or upwelling of subsurface waters of higher salinity. It is significant for this interpretation that the consistently positive trend in salinity levels out at ~ 150 m.

There is supporting evidence for these Bermuda results as similar trends are seen in other data. Zverev⁵ analysed long-period surface water temperature anomalies in the North Atlantic from weather ship data, 1948-68. At Ocean Weather Station Echo which lies east of Bermuda he found a consistent downward trend of $\sim 2^\circ$, from 1954 to 1968. Fieux and Stommel⁶ studying sea-surface temperature records from ships south and east of Bermuda

showed consistent decrease in sea-surface temperature since 1954. Our record at the Panulirus Station not only confirms consistent trends for sea-surface temperature but follows them down the water column and extends them to other parameters.

Full consideration in any future analysis of climatic trends in the North Atlantic should be given to this unique mid-Atlantic monitoring station.

R. POCKLINGTON

Atlantic Oceanographic Laboratory,
Bedford Institute of Oceanography,
Dartmouth, Nova Scotia,
Canada B2Y 4A2

1. Kukla, *et al.* *Nature* **270**, 573-580 (1977).
2. Schroeder, E. & Stommel, H. *Progr. Oceanogr.* **5**, 31-40 (1969).
3. Pocklington, R. *J. geophys. Res.* **77**, 6604-6607 (1972).
4. Pocklington, R. *Variability in the Ocean off Bermuda, Rep. ser. BI-R-72-3* (1972).
5. Zverev, A. A. *Oceanology* **12**, 182-186 (1972).
6. Fieux, M. & Stommel, H. *J. mar. Res.* **33** Suppl. 83-95 (1975).

Ozone in London

STEWART *et al.*¹ presented "a model for ozone concentrations at" a "Central London background site (Endell Street)". Ball² has summarised ozone levels for the summer of 1976 at County Hall (approximately 1.5 km from Endell Street), and it seems reasonable to test the model using this data together with London Weather Centre observations.

On 26 and 27 June 1976 peak hourly ozone concentrations of 21.0 and 21.2 p.p.h.m. (parts per 10⁶) were recorded at County Hall. Using the notation of Stewart *et al.*'s model

$$(O_3)_t = 0.68(O_3)_{t-1} + 0.38T_t^{0.37}I_t^{0.28}H_t^{-0.36}$$

On 26 June: $(O_3)_{t-1} = 16.6$ p.p.h.m.; $T_t = 34.8^\circ\text{C}$; $I_t = 650$ mWhcm⁻²; $H_t = 28\%$; therefore, $(O_3)_t = 13.5$ p.p.h.m. The actual value was 21.0 p.p.h.m.

On 27 June $(O_3)_{t-1} = 21.0$ p.p.h.m.; $T_t = 33.7^\circ\text{C}$; $I_t = 623$ mWhcm⁻²; $H_t = 31\%$; therefore, $(O_3)_t = 13.9$ p.p.h.m. Actual value, 21.2 p.p.h.m.

The model underpredicts these high values by approximately one third, and is therefore of little use as a predictor of high ozone concentrations. This is probably because the model cannot distinguish between locally generated and imported ozone³.

In order to examine the assumptions of the model, the original data used in its derivation was kindly lent to me. Several interesting points emerged:

(1) The axes in Fig. 2 of Stewart *et al.*'s paper are the wrong way round. In fact their model underpredicts high observed values as shown above. Also the standard error given by Stewart *et al.* in the text for their model ($\pm 2.1 \cdot 10^6$) is incorrect. Figure 2 shows that the standard error in logarithmic coordinates is 0.75 (units log_e). Now anti-log_e 0.75 ≈ 2.1 which is the number given by Stewart *et al.*, but

Table 1 Simple correlation coefficients between the independent variables

	$\ln \{O_3\}_{t-1}$	$\ln T_t$	$\ln I_t$	$\ln H_t$	$\ln W_t^*$
$\ln \{O_3\}_{t-1}$	1.00	0.38	0.28	-0.23	-0.09
$\ln T_t$		1.00	0.47	-0.26	-0.31
$\ln I_t$			1.00	-0.68	-0.04
$\ln H_t$				1.00	0.12
$\ln W_t$					1.00

Only 427 cases were actually used in the comparison. Any cases with missing data were eliminated completely from the analysis.

* W_t is the average wind speed (ms^{-1}) between 1000 h and 1600 h at the London Weather Centre.

when converted into arithmetic units this is the factor by which the mean value must be multiplied or divided by to find the range for one standard error. In fact the standard error lines shown in Fig. 2 are apparently drawn at 2 standard errors from the mean. If this is the case then one standard error ≈ 0.375 (units $\log_e$) or a factor of 1.45 in arithmetic units. Thus the actual limits on the mean value are +45% and -31%. Stewart *et al.* state a confidence limit of $\pm 26\%$ for predicting elevated levels above 8.0 p.p.h.m. This is incorrect not just because of the above reasoning but also because the standard error lines should not be drawn parallel to the regression lines as shown in Fig. 2. The standard error is a minimum in the 'middle' of the observed range and increases to either side. In other words for elevated ozone levels the standard error will be greater than for the mean value. (2) The meteorological predictors used in the model should strictly speaking be independent. However, Table 1 shows that they are far from independent. Multicollinearity can mean that computed regression and correlation coefficients vary markedly from sample to sample. One test for multicollinearity is to randomly split a data set into two values and compare the computed coefficients. For the ozone data it was thought that it might be more instructive to split the data into days when the peak hourly concentration was above and below the background peak level of ≈ 4.0 p.p.h.m. The results of this analysis are discussed in detail elsewhere³. Suffice it to say that the regression coefficients for temperature, insolation and wind speed (neglected by Stewart *et al.*) were different for the two data sets as shown in Table 2 below. The strong positive correlation between low ozone levels (less than 4.0 p.p.h.m.) and wind

speed, may be because higher wind speeds result in lower concentrations of locally emitted nitric oxide, and hence a smaller loss of background ozone. On the other hand, in light winds a city such as London will act as an effective ozone sink, and very little will be brought in from outside the city. In stronger winds, natural background levels are more likely to persist because of the import of natural levels from outside the city, even though the city itself is still acting as a sink. Whatever the mechanism involved it is obvious that wind speed should not be ignored.

(3) One must be extremely careful in any statistical analysis to fulfil all the assumptions of the statistical model used. I do not present my equations given in Table 2 as substitutes for the model of Stewart *et al.*, but merely to illustrate the point that this type of 'black box' analysis is not applicable to the prediction of ozone levels in London.

JOHN E. THORNES

Department of Geography,
University College,
London, UK

1. Stewart, E. J. *et al.* *Nature* 263, 582-584 (1976).
2. Ball, D. J. G.L.C. Scientific Branch. Rep. No. E59/EA/R49 (1977).
3. Thornes, J. E. *Prog. phys. Geog.* 1, 506-517 (1977).

WILLIAMS AND SULLIVAN REPLY—The first of Thornes' main points concerns the underprediction of ozone levels on several days during the summer of 1976. In response to this we feel that some general comments on statistical models are appropriate. Any statistical model is historical, in the sense that its range of applicability to future conditions is limited by the range of conditions used in its formulation. Given this fact and bearing in mind the abnormal meteorological

conditions prevalent in the summer of 1976, it is not surprising that ozone levels were underpredicted on some days in that period. The low prediction is most probably due to considerations such as these, rather than as Thornes suggests to any distinction between locally generated and imported ozone which is not appropriate to a gross statistical model such as the one in question. For example, the summer of 1977 has proved to more closely resemble the period for which the model was formulated than did summer 1976. For instance, on one of the days of highest ozone levels, 28 May 1977, the model predicts a maximum value of 8.9 p.p.h.m. compared with an observed 9.5 p.p.h.m.

On the point of the error of prediction and confidence limits we agree with Thornes comments, but would mention that the definition of standard error used in the note was 1.96 ($=t_{5\%}$) times that used by Thornes. The axis labels in Fig. 2 should be interchanged.

The condition of the independence of the predictor variables has been fully recognised by workers in this field as being not strictly fulfilled so that all such analyses are performed with this in mind and all results are naturally subject to the extent to which, in practice, this is found to be a problem. Indeed in the majority of such regression analyses of pollutant/meteorology relationships it would be difficult to satisfy this condition fully.

The comment that the wind speed was neglected is not strictly correct as we stated that this variable was considered and was found not to contribute significantly, when all the ozone data were taken into account. The findings of Thornes are in agreement with this conclusion. As our purpose was to investigate the gross correlations of all the ozone data with meteorological parameters, it was not felt to be appropriate to analyse in greater detail in the original note.

Notwithstanding these considerations we feel that regression analyses can prove useful in highlighting the important factors in this type of problem as was the intention in our note, but would agree that considerable caution needs to be exercised in attempting to draw detailed conclusions from such studies particularly regarding in this case, mechanisms leading to elevated ozone levels. In the time that has elapsed since the note was published it has become more apparent that more detailed physical models of ozone formation which treat atmospheric chemistry as well as the diffusion and advection of pollutants, are more likely to provide a better understanding of such mechanisms.

M. L. WILLIAMS
E. J. SULLIVAN

Department of Industry,
Warren Spring Laboratory,
PO Box 20, Ginnels Wood Road,
Stevenage, Herts, UK

Table 2 Correlation coefficients and regression equations for $\{O_3\}_t < 4.1$ and ≥ 4.1 and for the total data set

For ozone	$\ln T_t$	$\ln I_t$	$\ln H_t$	$\ln \{O_3\}_{t-1}$	$\ln W_t$	No. of cases
≥ 4.1	0.47	0.23	-0.30	0.38	-0.34	220
< 4.1	0.09	0.36	-0.24	0.27	0.24	207
All	0.47	0.55	-0.47	0.55	0.13	427

					Multiple r	Standard error of mean (%)
≥ 4.1	$\{O_3\}_t = 5.7 \{O_3\}_{t-1}^{0.19} T_t^{0.44} I_t^{-0.007} H_t^{-0.3} W_t^{-0.21}$				0.62	+29%; -22%
< 4.1	$\{O_3\}_t = 0.75 \{O_3\}_{t-1}^{0.19} T_t^{0.001} I_t^{0.2} H_t^{-0.12} W_t^{0.24}$				0.50	+38%; -28%
All	$\{O_3\}_t = 0.69 \{O_3\}_{t-1}^{0.18} T_t^{0.35} I_t^{0.26} H_t^{-0.34} W_t^{-0.03}$				0.71	+48%; -32%

reviews

Compact history of meteorology

Gordon Manley

The History of Meteorology to 1800.
By H. Howard Frisinger. Pp. 148 (Neale
Watson: New York, 1977). \$15.

THIS publication in the Historical Monograph series of the American Meteorological Society is a short but useful introductory assemblage, a straightforward compact history that abridges much that is familiar to students of the scientific aspects of our European culture. We find plenty about Aristotle and Descartes, Galileo, Von Guericke, Hooke, Halley and Hadley, Pascal, d'Alembert and Euler. The author inclines to short statements with little discussion, but has made good use of such well-known earlier sources as Hellmann, Cajori, Napier Shaw, Dampier, Wolf and more recently, Middleton on the development of meteorological instruments. Needham on Chinese science is not mentioned.

Of the eight chapters, the first three cover the period of speculation. Brief accounts of the contribution of earlier Greek philosophers are followed by ten pages on Aristotle's *Meteorologica*. Thereafter almost two millennia are regarded as the dark before the dawn, with comments on Seneca and Pliny, Isidore and Bede, Adelard and Roger Bacon, the first hints of the individual maintenance of regular daily observations.

In the age of mechanical clocks, spectacles, the compass, the astrolabe and the accounting of mercantile transactions, meteorology was evolving. The author does not comment on the association with the expansion of knowledge of the world that arose with the Crusades, Marco Polo, and the first universities. For many the intriguing question remains: whether the keenly observant craftsmen-sailors of Northern Europe did not have a recorded lore of their own—effective but, like the portolan charts, tardily acknowledged by the churchmen? Or was it, like the sagas, orally transmitted? Who began measuring and comparing, besides counting and recording? We are then led up to Cardano's *De Subtilitate* (1550) as the beginning of the end of Aristotle, and the observations of Tycho Brahe and Kepler, still disposed to link their astronomy with meteorology.



d'Alembert (1717–83)



Halley (1656–1742)



Euler (1707–83)



Pascal (1623–62)

Five later chapters then cover the dawn of scientific meteorology, and are centred around the themes of the thermometer, the barometer, the hygrometer and other instruments. We are reminded of the contributions of Galileo, Torricelli, von Guericke, and the Accademia del Cimento. Appropriate and compact comment is given to Descartes and the scientific method. Thereafter, a chapter is given to the organisation, assembly and discussion of meteorological observations through Hooke, Jurin, the Mannheim Society; and a final chapter emphasises the contributions of Halley, d'Alembert and Euler. The developing applications of mathematics in the discussion of changes of pressure with height and the observations that led to the beginnings of the physics of gases and water vapour are touched on.

Meteorology is the study not only of the motion, but also of the phenomena of the atmosphere; the author might have said more about the phenomena. It is surprising to find no mention of Franklin, or for that matter John Lining of Charleston. It is welcome to be reminded of one of the less familiar figures, the New England pioneer Isaac

Greenwood, Professor of Mathematics at Harvard in 1727. Mention might have been made of the difficulties facing his predecessors; Derham in a letter to the Royal Society was appreciative of Robie's New England observations (1715–22), but noted that they lacked barometer and thermometer readings as such instruments were not available in the colony, although they could be bought in London before 1690.

More could have been said of the contributions of the biologists and medical men to knowledge of the characteristics of the atmosphere; the effects of radiation, of what was meant by frost, what properties were really being measured and how they could be compared. The importance of eighteenth-century mountain exploration and later, the balloon; theories of the formation of rain; and the contributions of the craftsmen in such matters as annealing glass and boiling mercury might also have been mentioned.

Plotting the behaviour of the air, both in space and time, was considered even as early as Halley; and this can be seen also from the correspondence

of such men as Scheuchzer and Derham. We are told that Lambert in Germany seems to have been the first to present meteorological data in the form of graphs rather than in tables; but such plotting can be found half a century earlier—for example, by Cruquius, showing the course of a year's observations of pressure (*Phil. Trans. R. Soc.* 33 (no. 381), 1724).

Halley's approach to global meteorology (1686) on the trade wind circulation is appropriately discussed and his celebrated map is reproduced (pp126–7); it seems supererogatory to show the evident copy of Halley's map that was published by d'Alembert (p131). There are some infelicities: strata is a plural on p16, but is used as a singular on p17; on p3 some may question the phrase "the cyclic movement of water falling

from the sky in the form of rain and condensing back into the sky"; and on p78 work by Hooke is wrongly ascribed to *Trans. R. Soc. Edin.* long before it was founded.

There is admirable provision of references for those who wish to go further, and many welcome illustrations, notably that of Wren's weather clock. As a short history, the book is agreeable as far as it goes, although the author's commendable regard for the dynamic aspects together with his evident effort at brevity has led to the omission of a good deal that is germane to the evolution of the science as it stood in 1800. □

Gordon Manley was Professor of Environmental Sciences at the University of Lancaster from 1964–68.

Gap junctions and chemical synapses

Receptors and Recognition. Series B. Vol. 2: Intercellular Junctions and Synapses. Edited by J. D. Feldman, N. B. Gilula and J. D. Pitts. Pp. 246 (Chapman and Hall: London, 1978.) £15.

THIS book sprang from a highly successful meeting organised in 1976 by one of the Editors, J. D. Feldman, and covers the two main major areas of interest at that meeting: gap junctions and chemical synapses.

The fact that the gap junction is still (as Wolpert puts it) "a structure in search of a function" inevitably pervades the first chapters. This is a reflection of the field, rather than the authors, who between them provide a good statement of current knowledge, but few new insights. The chapters either provide relatively straightforward accounts (structure by Gilula; permeability by Bennett) or range widely (growth control by Pitts; developing systems by Wolpert). The latter two chapters encompass those areas in which direct cell to cell communication (probably mediated by gap junctions) can most easily be seen to be necessary.

Even so, experimental evidence that intercellular communication of the type mediated by gap junctions plays an essential role is lacking. It is difficult to agree with Pitts' assertion that junctional permeability has been convincingly shown to be the same *in vivo* as *in vitro*. The few experiments which have been done *in vivo* (cited in another review by the same author) do not allow such a general conclusion, because it is not yet possible to do the same kind of experiments *in vivo* as *in vitro*. Wolpert presents a more balanced

view, perhaps because he is not personally involved in the experimental investigation of gap junctions, yet is concerned with their possible role during early development.

Sheridan brings together information on junction formation from scattered sources. He also takes a careful and critical look at the experiments of Loewenstein and his group on the possible role of calcium in controlling the permeability of the gap junction.

The synapse section begins with a comprehensive review by A. Matus on the present state of the structure of chemical synapses and current progress with isolation of its protein components. J. D. Feldman and C. R. Slater tackle the problem of specificity of synapse formation in completely different ways. Feldman takes a broad overview of the process, drawing freely on an analogy between the neurone searching for its contact and the migrating bird seeking its destination; whereas Slater restricts himself to the more discreet topic of the mammalian neuromuscular junction. They are both, in their different ways, interesting and instructive. The first part of Kelly's chapter on cholinergic processes clearly summarises the present situation, although the relationship between the latter part and the rest of the book is difficult to see.

Should one buy this book? Libraries yes, for it provides a useful introduction for the third-year undergraduate and newcomer to the field, but I cannot see individuals buying a book which, one hopes, will soon become out of date as research into these two topics advances.

Anne Warner

Anne Warner is Senior Lecturer in Anatomy and Embryology at University College, London, UK.

Mass spectroscopy

Principles of Field Ionization and Field Desorption Mass Spectrometry. By H. D. Beckey. Pp. 335. (Pergamon: London and New York, 1978.) £19.50; \$35.

THIS book was originally intended to be a second edition of the author's *Field Ionization Mass Spectrometry* which was largely written in 1969. However, developments in the field in subsequent years convinced the author that he should produce an essentially new volume dealing with the subject under five main headings, which appeared to represent the growth areas. These are: theory of field ionisation (FI) and field desorption (FD), experimental methods, kinetics and mechanisms of decomposition of field ions in the gas phase, and qualitative and quantitative analysis with field ionisation and field desorption mass spectrometry.

After dealing with the theory of FI and FD, the next chapter goes on to describe the techniques of FI and FD, and in particular the design and performance of sources. This chapter should be of value to anyone intending to modify a conventional mass spectrometer to encompass these techniques. The advantages and disadvantages of tips, blades and thin wires are discussed in detail. Problems of resolving power and sensitivity are also broached. The third chapter again deals with theory, this time being more concerned with ionic decomposition under high field conditions. The energetics of such decompositions is discussed in some detail, and methods of determination of the appearance potentials of fragment ions are reported.

The kinetics of ion decomposition consequent upon FI form the subject matter of chapter four. This is an area of particular interest, as the time scale of the decompositions can be as short as 10 ps. In particular the normalised rate constant $k(t)$ (sometimes called the phenomenological rate constant) is dealt with in some detail. A number of examples are given of the application of the theory to specific unimolecular decompositions.

The final chapter deals with the analytical aspects of FI and FD mass spectrometry. Applications of the recently developed field of alkali ion attachment are discussed.

This book is a worthy successor to the earlier volume and must have a place on the shelves of any one working or intending to work on FI or FD. There are nearly four hundred references, which should enable the newcomer to find his way around the field. The only criticism I would make is of the unjustified right-hand margins, which I find most distasteful.

Allan Maccoll

Allan Maccoll is Professor of Chemistry at University College, London, UK.

British Quaternary studies

British Quaternary Studies: Recent Advances. Edited by F. W. Shotton. Pp. 298. (Oxford University Press: Oxford, New York and London, 1977.) £10.

All Quaternary scientists stand in Professor F. W. Shotton's debt for his organising and hosting the Tenth Congress of the International Union of Quaternary Research (INQUA) in Birmingham last August. In conjunction with this meeting, Professor Shotton edited *British Quaternary Studies: Recent Advances*, and copies were distributed to the delegates. Twenty-three authors contributed 20 well-paced brief reviews on a broad range of Quaternary topics from tills (Shotton *et al.*), sea levels (Mitchell), and loess (Catt) to molluscs (Kerney, Norton), beetles (Coope), vegetation (Godwin, Birks), and man (Molleson, Wymer); and from detailed descriptions of local stratigraphic sections (Funnell and West) to the results of computerised glaciological models (Boulton *et al.*). Most articles summarise work within Britain and describe events over several glacial-interglacial cycles. McCave *et al.* and Shackleton describe work in the North Sea and the world's oceans.

The context in which this book was published suggests that it is aimed at an international audience and is designed to represent recent British Quaternary studies. Does it meet these goals?

The most successful article for an international audience is Mitchell's discussion of British sea-level changes in the context of oxygen-isotope variations within deep-sea cores. His correlations between the continental record and the isotope curve may be modified in the future, but the local British changes are well described within the framework to which all long-term Quaternary events from all continents must be tied.

Most other articles describe British phenomena in terms of British stratigraphical terms (for example, Hoxnian, Ipswichian, Devensian, and so on). This practice requires the non-British reader to seek tables that correlate the British terms to those used on other continents or that link the British events to an absolute time-scale or the deep-sea record. No table of this type appears in *British Quaternary Studies*. Although uncertainty and controversy are always associated with tables of this type, Professor Shotton has constructed such a table in previous publications (for example, G. F. Mitchell *et al.*, *Geol.*

Soc. Lon. Spec. Rept., 4, 1973) and might have directed the reader to this publication in his introduction. A map locating all sites mentioned in the book would also have helped.

For the timescale of the past 100,000 yr to 2 Myr, the British work is well reviewed. Fossil remains and geomorphological features are described in the context of glacial and interglacial periods in Britain. The establishment of this Quaternary sequence of cold and warm periods is one of the most important contributions that Britain has made to Quaternary science. Focussing the reviews primarily on this timescale, however, has led to the exclusion of several types of Quaternary research in which British scientists have pioneered and hold international reputations.

For instance, Molleson and Wymer ably review the long-term archaeological finds in Britain, but no article

describes the detailed interactions between man and the environment that have been documented palynologically and archaeologically for Neolithic and more recent times. No review appears of the recently developed palaeomagnetic techniques for studying lake sediments, nor are any of the excellent palaeolimnological and geochemical studies of lake sediments described. The advances in tree-ring research are also missing. These omissions restrict the book to helping mainly those scholars already versed in the British Quaternary who wish to update their knowledge of some but not all aspects of this field.

Thompson Webb

Thompson Webb III is Associate Professor of Geological Sciences at Brown University, Providence, Rhode Island, and a Visiting Fellow (1977-78) at the Botany School, University of Cambridge, UK.

Microbial interactions

Receptors and Recognition. Series B. Vol. 3: Microbial Interactions. Edited by J. L. Reissig. Pp. 436. (Chapman and Hall: London, 1978.) £20.

Two concurrent series are being published on *Receptors and Recognition* in biological processes: one (Series A) is devoted to wide-ranging articles and the other (Series B) deals with detailed consideration of a specific area. This particular volume consists of nine chapters written by experts in their fields, with an overview contributed by the volume editor, Professor J. L. Reissig.

The first chapter, on "Aggregation and Cell Surface Receptors in Cellular Slime Molds", highlights the central role of cyclic AMP in inducing a population of independent amoebae to come together (cell signals) to form slug-like entities. The role of specific cell surface receptors in the final phase of aggregation (called "docking" by the author) is clearly presented.

The next five chapters deal with these two themes, of response to signals and cell surface receptors in bacterial systems. The first of these chapters concerns chemotaxis, and is followed by articles on phage and colicin receptors as constituents of specific transport systems, attachment to animal cells, the binding and entry of DNA in transformation, and redefinition of the mating phenomena in bacteria. The first two chapters of this group are well written and describe interesting areas of work. The chapter on attachment of bacteria to animal cells is somewhat disappointing in that the field is so

rapidly moving that it seems rather dated. The last two contributions, on transformation and on mating phenomena, were really quite exciting to read and must be counted as the high spots of the volume.

The topic of mating is extended to the eukaryotic organisms in chapters dealing with mating in the *Saccharomyces cerevisiae* and *Chlamydomonas* systems. The concepts of cell to cell interactions in the Ciliata is explored in the next chapter. The volume editor draws together the underlying ideas in the previous chapters in a thought-provoking final essay, and supplies a useful Thesaurus of Microbial Interactions.

The major omission in the book is that only single-species systems are examined. It would have been useful to consider how mixed systems behave, because in most natural environments mixed communities of microbes play a dominant role. The papers dealing with the bacterial systems all refer briefly to the fact that the growth environment of the organism has important effects on the system under study. However, this parameter is often not considered by many authors and it is clear that much understanding would come if the dynamic nature of bacterial surfaces was fully recognised.

The book is important as it brings together many viewpoints in this rapidly expanding field of microbial interactions. It should be read by all those interested in the topic.

D. C. Ellwood

D. C. Ellwood is Head of the Division of Biochemistry at the Microbiological Research Establishment, Porton, and Honorary Professor of Environmental Sciences at the University of Warwick, UK.

Steroid hormone receptors

Receptors and Hormone Action. Vol. 2. Edited by B. W. O'Malley and L. Birnbaumer. Pp. 602. (Academic: New York and London, 1978.) \$46; £32.65.

As the editors mention, there has been a tenfold expansion in research on hormone action. Because receptors are the central issue of understanding how hormones regulate the activity of their target cells, this "explosion" is also reflected in published research on hormone receptors. In this second of a three-volume series, 600 pages are devoted to steroid hormone receptors (if one accepts vitamins A and D as honorary members of the steroid club). The main theme to emerge repeatedly in the 19 chapters by different authors is the confirmation of Jensen's and Gorski's original two-step mechanism, whereby a cytoplasmic protein combines with the hormone, the complex is then translocated into the nucleus where it initiates the target cell's physiological response.

The book begins with an account by Clark *et al.* on the relationship between the binding of oestrogen, and its agonists and antagonists, to uterine nuclear receptor and nuclear RNA synthesis followed by growth of the tissue. They conclude that the mere nuclear accumulation of the hormone-receptor complex is not enough to cause uterine growth but only if the receptor-oestrogen complex resides in the nucleus for a substantial period of time. The temporal requirement is explained by the conversion of the receptor complex from one state to another. In the next chapter, Notides discusses the possibility of the cytoplasmic oestrogen receptor existing in conformationally distinct dormant and active forms, the transformation being the key to hormonal action. Stormshak *et al.* deal with oestrogen and DNA synthesis, a hormonal effect most likely to be remote from the primary site of action.

Four chapters are exclusively devoted to androgen receptor and action, three to glucocorticoids, two to progesterone and one each to mineralocorticoid and vitamins A and D receptors. McEwen, McGuire *et al.* and Westphal also cover many of these hormones in their chapters on receptors in neuroendocrine tissue, breast cancer, and circulating steroid-binding proteins, respectively. Not surprisingly there is much repetition, but many novel ideas also emerge. To cite a few, Liao's group suggest that the binding of androgen-receptor complex to ribonucleoprotein particles in prostatic

nuclei might mean that the complex not only regulates transcription but also RNA processing and packaging. Baxter and Ivarie discuss some genetic approaches to studying glucocorticoid receptor in cultured lymphoma cell variants showing steroid resistance, a study initiated by the late Gordon Tomkins.

The importance of genetic approaches has not been fully realised and it is a pity that the topics of testicular feminisation syndrome and ecdysone receptors in insect cells have not been included. The genetical approach is also cited by Mainwaring as one of the "desirable trends in future research", the others being the question of purification of the receptor and the development of new experimental systems and new technology for a functional study of the receptor. Indeed, the true physiological relevance of all work on 'receptors' will only emerge when some means will be found to link the mass of

data on the binding of steroid hormones to various cellular constituents, on the one hand, and the variety of biochemical reactions they provoke in their target cells, on the other.

For the general reader a chapter, preferably written by one of the editors, summarising the common themes as well as the uniqueness of some hormone receptors, would have been most useful. But this book is not for the general reader. It is a timely and valuable treasury of information for those already working on receptors and actions of steroid hormones. It will also be useful to those interested in steroid biochemistry, endocrinology, regulation of protein synthesis and nucleocytoplasmic interactions.

J. R. Tata

J. R. Tata is Head of the Laboratory of Developmental Biochemistry at the National Institute for Medical Research, London, UK.

Southern perspective on climatic change

Climatic Change and Variability: A Southern Perspective. Edited by A. B. Pittock, L. A. Frakes, D. Janssen, J. A. Peterson and J. W. Zillman. Pp. 455. (Cambridge University Press: Cambridge, New York and London, 1978.) £17.50.

This book arose out of a conference on the theme "climatic change and variability, with particular reference to the Australian or Southern Hemisphere region" which was held at Monash University in December, 1975. It consists of edited versions of invited review papers, supplemented by a small selection of heavily edited versions or amalgams of contributed papers. The contributors are nearly all acknowledged world experts in their respective fields and the book is intended as a university level text suitable for introductory courses in the Earth sciences. Among the topics discussed are: a description of the physical basis of climate; the long- and short-term climatic record; models and mechanisms of climatic change; climatic forecasting; and the economic social and political implications of climatic change.

As the authors are all experts in their particular fields, *Climatic Change and Variability* contains information on much of the latest thinking on climatic change. The disadvantage of this multi-author book is that it lacks a uniform style, and the general level of treatment is somewhat uneven. It is a little too condensed to be a successful introductory text, and there is a tendency to assume that the reader is familiar with most of the climatological and meteorological con-

cepts being discussed. For example, G. B. Tucker gives an excellent summary of the general circulation, but his description of the energy cycle is much too terse for use by a student who is not already familiar with this particular concept. There is a tendency for the authors to overlap in their treatment of certain subjects. Thus, both L. A. Frakes and R. W. Fairbridge discuss long-term geological changes in climate. There are also some odd oversights, as for example when R. G. Barry produces a diagram showing the world's greatest observed rainfalls which has been out-of-date for many years.

The book ends with a report of a panel discussion on possible future climatic trends. H. Flohn comments that the probability of a transition to the type of climate leading to an ice age in the next 100 years is less than 1%, but that the probability of large-scale human interference with climate leading to a global warming next century is great. Indeed it is a pleasant change to see a climatological textbook which does not take the extremely pessimistic view that mankind is doomed by the weather to suffer the deprivations of a new ice age. The final chapters do stress the importance of climate to the economy and include an interesting section by R. A. Bryson on "The Cultural, Economic and Climatic Records".

Climatic Change and Variability is well produced and the equal of any book published in the past year in the field of climatic change. I recommended it to all environmental science students, especially those living in Australia and New Zealand.

J. G. Lockwood

J. G. Lockwood is Senior Lecturer in Geography at the University of Leeds, UK.

obituary

A. D. Gardner

PROFESSOR Arthur Duncan Gardner, a bacteriologist whose career was centred in Oxford, died on 28 January 1978 at the age of 93.

He was educated at Rugby and University College, Oxford, where he originally read law before changing to medicine. He qualified from St Thomas's Hospital in 1911, took his Fellowship of the Royal College of Surgeons, was made a lecturer in pathology, and was awarded the Radcliffe Travelling Fellowship in 1914. During the first world war he served for a time with the British Red Cross in France, and in 1915 was appointed to the directorship of the Standards Laboratory that had been newly established at Oxford by the Medical Research Council.

One of his chief functions was to supply the military and naval forces with reagents for the diagnosis of typhoid and paratyphoid fevers. This was made possible by adopting the standard killed agglutinating suspensions devised by Georges Dreyer in place of the usual living organisms. These were soon supplemented by six dysentery suspensions with their corresponding antisera for use in Salonica. The discovery of flagellar and somatic antigens and of specific and non-specific phases in organisms of the *Salmonella* group showed that the preparation of standard suspensions was far more complex than Dreyer had foreseen, and demanded the use of suspensions containing only the somatic antigen. Reagents for the diagnosis of brucella infections, typhus fever, and meningitis followed in due course. In 1936 Gardner was made Reader with the title of Professor, and in 1939 gave place as director of the Standards Laboratory to Lt-Col. R. F. Bridges.

During the thirties, Gardner with P. H. Leslie greatly clarified the antigenic structure of the whooping-cough bacillus by defining four phases, which they named I, II, III, and IV. With Venkatraman he performed a similar function in showing the occurrence of six subgroups in the cholera-*el*-non-agglutinable group of vibrios, characterized by different O antigens, only the O antigen of subgroup I being specific for the diagnosis of cholera.

Gardner will also be remembered for his participation in the original work, with Florey and Chain, on penicillin at

Oxford. Not only was he responsible for determining the susceptibility of different bacterial species to penicillin, but he was the first to give a clue to the mode of action of penicillin by demonstrating its interference with growth and division, rather than, as was commonly supposed, by killing the mature organisms.

A new, and final phase in his career opened in 1948 with his appointment to the Regius Chair of Medicine at Oxford. (This was the first of three appointments in which laboratory workers attained a high office usually held by practising physicians, the others being that of Sir Lionel Whitby, a haematologist, to the Regius Chair of Medicine at Cambridge, and of Sir Charles Dodds, a chemical pathologist, to the Presidency of the Royal College of Physicians of London—surely a unique conjunction and a feather in the cap of laboratory medicine.) This new task, undertaken at a difficult time, when the future of the clinical school was at stake and the National Health Service had just come into being, afforded greater scope for Gardner's personal qualities of charm, tact, and determination. In his six-year tenure of office ending with his retirement in 1954 at the age of 70 he achieved much and gained the respect and admiration of all those with whom he had to deal.

L. P. Garrod
G. S. Wilson

Rudolf Kompfner

PROFESSOR RUDOLF KOMPFFNER, the electrical engineer and physicist, died on 3 December, 1977.

He was born in 1909 in Vienna where he trained as an architect. After coming to England in 1934 and practising until 1941, he went to the Physics Department of the University of Birmingham where he worked with the Admiralty group on microwave radar. While there he invented the travelling wave tube (TWT), a microwave amplifier of great importance.

In 1944 he joined the Clarendon Laboratory in Oxford. My colleague Don Walsh recalls Rudi's visits to the Services Electronics Research Laboratory in Baldock with a view to developing a medium power X-band TWT. Don's first attempt to make this tube floundered as it proved impossible to get any of the cathode current to complete the journey through to the collector. Rudi recounted how he had had the same problem with his first

TWT. He had tried very hard to measure the current reaching the end of the helix structure eventually borrowing the most sensitive galvanometer in Birmingham University to measure it. He eventually realised that stray magnetic fields were affecting the beam focusing so he surrounded the tube with screening and almost immediately burnt out the most sensitive galvanometer in Birmingham University. In fact with his very first tube he was able to measure gain and confirm its dependence on electron velocity.

In 1951 Rudi left Oxford to join Bell Telephone Laboratories. Here he invented the backward-wave oscillator, his second major invention, and was responsible for the Telstar communications satellite and important work in radio astronomy. He was also involved in much work in the fields of lasers and optical communications.

In 1973 he retired from Bell Laboratories and for three years spent half of each year as Professor of Engineering at Oxford University and the rest of his time as Professor in the Applied Physics Department of Stanford University. At Oxford he initiated work in various applications of lasers including a scanning optical microscope, optical fibres and holography, and it is in this research that I first became acquainted with him. I feel very privileged to have worked with such a great man. He had a rare quality of being able to inspire those around him with his enthusiasm, and on a number of occasions I was very late home after getting thoroughly engrossed in a long discussion about some technical matter. His analyses relied heavily on graphical constructions, which presumably stemmed from his architectural training, and he kept beautiful notebooks with details of all his work. He spent most of the day just sitting at his desk inventing (as he once said "Every now and then I have a little idea") although he was also a careful experimentalist. Eventually, because of our tax laws, he was forced to spend only three months in England each year although he still continued to provide us with guidance and plenty of ideas. At Stanford he worked on other novel methods of microscopy including the scanning acoustic microscope which uses acoustic radiation to produce sub-micron resolution.

He was a fellow of the former

Physical Society (1949) and of the Institute of Radio Engineers (1950), a member of the National Academy of Sciences (1968) and the National Academy of Engineering (1966) in America. He received an honorary Doctor of Engineering degree from the University of Vienna (1965) and an honorary Doctor of Science degree from the University of Oxford (1969). He received the Duddell Medal of the Physical Society (1955), the Sarnoff Medal of the Institute of Electrical and Electronic Engineers (1960), and the Ballantine Medal of the Franklin Institute (1960). In 1973 he was made a Fellow of All Souls and in 1976 he was awarded the U.S. President's Award for Achievement in Science.

He was a most likeable person with a strong sense of humour ("I might remark that there is no harm in getting 'expert' advice. But don't take it"). He had a heart attack ten years ago on a mountain top in Vermont and used those ten years to the maximum advantage, accomplishing much more than most people would dream of doing in a lifetime.

He leaves a wife and two children, and was very pleased to have recently had a grandchild.

C. J. R. Sheppard

H. von Klüber

THE death of Dr Harald von Klüber on 14 February 1978 deprives solar physics of one of its finest observers. Von Klüber was born at Potsdam on 6 September 1901, into a family which for generations had held high office in the civil, military and diplomatic services. Educated at the Universität und Technische Hochschule, Berlin, he took his doctorate there and became Professor of Astrophysics at Potsdam in 1941. Soon after the end of the war he and his wife fled to Switzerland taking with them only a few necessities in a suitcase. At that time Professor Redman was seeking new staff at the Cambridge Observatories, and von Klüber was welcomed there in 1949 as the new Observer in Solar Magnetism; he and his wife occupied a cottage in the grounds of the Observatories and immediately became much liked members of the community. This was the beginning of the second phase of a fruitful career.

Von Klüber's earliest work was concerned with stellar physics, but he soon became interested in solar studies, inspired by ten Bruggencate and Freundlich, and by the advanced equipment available at Potsdam which was then one of the great centres of solar physics. At that time there was a resurgence of interest in the theory of formation of absorption lines in solar and stellar spectra, following the work

of Schwarzschild, Milne and Eddington, and others. The observer's contribution to this lay in the accurate measurement of solar line profiles, and von Klüber took his full part in this, working in part with Houtgast.

Another contemporary problem was the measurement of the gravitational deflection of light by taking photographs of a star field near the solar limb at a time of total solar eclipse. This is an astrometric problem of tremendous difficulty which had first been tackled, with some success, by Eddington and his collaborators at the 1919 eclipse, but confirmation of the 1919 results was desirable. This problem started von Klüber's interest in solar eclipse expeditions, beginning with the 1929 eclipse in Sumatra, and continuing after the war with Redman's encouragement, when he took part in, or was the leader of, no less than five expeditions. His interest later shifted to observation of the solar corona, and especially its optical polarisation, the interpretation of which was then causing some difficulty.

On coming to Cambridge he resumed his studies, started in Potsdam, of the local and general magnetic fields of the Sun. In collaboration with D. W. Beggs he built a fine solar magnetograph which they later successfully transferred to a good site in Malta. The station was closed down when von Klüber retired in 1971, and the von Klübers returned to the family home in Baden Baden.

Von Klüber was a meticulous worker who was devoted to his subject. A master of the methods of classical optics, he was equally keen on new instrumental developments, which he utilised fully. He had an especial interest in photography, which he applied so successfully to his private archaeological studies. All that he did, he did well. He and Mrs von Klüber (who survives him) were greatly liked and admired by all who knew him.

D. E. Blackwell
D. W. Dewhirst

C. W. Davies

PROFESSOR Cecil Whitfield Davies, D.Sc., F.R.I.C., one of the most distinguished and influential electrochemists of his time, died on 1 March 1978, at the age of 82.

He was educated at St. Dunstan's College, Catford, and at the University College of Wales, Aberystwyth. After serving with the Army between 1915 and 1919, in France and India, he returned to Aberystwyth in 1920 as Assistant Lecturer. He first did research in organic chemistry and obtained his M.Sc. in 1923, but during this period he realised that his interests lay in physical chemistry. In 1923 he

began working with L. J. Hudleston on the electrochemistry of pure HF solutions, and obtained accurate values for the transport numbers of the various species present. This work led Davies to predict the concentration dependence of ionic mobilities, a prediction which preceded the first paper by Debye and Hückel on this subject.

In 1928 he left for Battersea Polytechnic, and soon built up a flourishing research school which became well known for the very precise measurement of the conductivities of electrolytes in aqueous solutions, and of the solubilities of sparingly soluble salts. Central to these studies was the establishment, on a quantitative basis, of the concept of ion association in 'strong' electrolytes; this was of major importance in all his subsequent work. During this period Davies first formulated his semi-empirical activity coefficient equation, which has since been of great value to electrochemists everywhere.

In 1944 he returned to Aberystwyth to take up the Chair of Chemistry, and once again found himself having to create a research school almost from scratch. During the years up to his retirement in 1960 he established in the department one of the leading schools of electrochemistry in the country. Important contributions were made by Davies and his collaborators in a number of fields, including conductivity, the kinetics of crystal growth, ion-exchange resins, and the significance of ion association in reaction kinetics.

In 1960 he returned once more to Battersea as a Senior Research Fellow, in which post he remained until the college, which had become the University of Surrey, moved to its campus in Guildford in 1969.

Davies held many honorary appointments during his career, including membership of the Royal Society Committee on Chemical Symbols, and he became the honorary secretary of the Chemical Society in 1936. As well as many research papers, he published three books. Two of these, *The Conductivity of Solutions* (1930, 1933), and *Ion Association* (1962), have had considerable influence in the development of modern electrochemistry, while the third, *Electrochemistry* (1967), is a very thorough, up-to-date yet concise, account of the principles of electrochemistry, written in the clear and readable style which was characteristic of all Davies's work.

He was a man of great kindness, intelligence, and integrity; he will long be remembered with affection throughout the scientific community, and sadly missed by a wide circle of friends.

D. Irwin Stock